12-2013

ARACHIDONIC ACID REVERSES THE PYRIPROXYFEN AND IBUPROFEN INDUCED TOXICITY IN DAPHNIA MAGNA

GAUTAM KUMAR GINJUPALLI
Clemson University, gautamvet63@gmail.com

ARACHIDONIC ACID REVERSES THE PYRIPROXYFEN AND IBUPROFEN INDUCED TOXICITY IN DAPHNIA MAGNA

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

By
Gautam Kumar Ginjupalli
December 2013

Accepted by:
Dr. William S. Baldwin, Committee Chair
Dr. Stephen J. Klaine
Dr. Cindy. M Lee
Dr. Peter. Van Den Hurk
Dr. Robert S. Cohen

ABSTRACT

Healthy reproduction and neonatal sex ratios of *Daphnia* are crucial to the health of the aquatic ecosystems in which *Daphnia* play principal roles in trophic transfer of nutrients. Combinations of environmental factors such as availability and quality of diet, overcrowding, hypoxia, reduced photoperiods and fall in ambient temperatures perturb normal parthenogenic reproduction and induce sexual reproduction through male production. Male production provides a mechanism for overcoming specific stressors such as overcrowding and overwinter pond desiccation. However, it also induces a decrease in the *Daphnia* population that could have adverse implications on the pond ecosystem that depend on *Daphnia*. Interestingly, *Daphnia* exhibit great phenotypic and reproductive plasticity in response to different environmental stressors. I hypothesized that arachidonic acid (AA), a dietary ω-6 fatty acid that accumulates in the ovary of *Daphnia* influences *Daphnia* reproduction and is important in counteracting the male production effects induced by environmental stressors. In this study, I investigated the male production effects of a juvenile hormone analog, the insecticide pyriproxyfen in *Daphnia magna* and examined how a diet rich or poor in arachidonic acid influences overall fecundity and amelioration of pyriproxyfen mediated sex ratio. Further investigation is focused on the novel nuclear hormone receptor HR97g. Based on higher expression of HR97g in the ovaries of mature *Daphnia* and inhibition by arachidonic acid in vitro, I thought that interactions of AA and HR97g are important in regulation of neonatal sex ratios. However, HR97g is only partially activated by pyriproxyfen in vivo and the significance of their interaction needs to be determined. In addition, AA is

metabolized to eicosanoids and I hypothesized that these molecules may play a role in male production. However, ibuprofen inhibition of AA did not perturb male production, but it did induce selective developmental abnormalities. In conclusion, pyriproxyfen is a juvenile hormone pesticide that induces male production in a time-dependent manner, and AA can in part ameliorate this activity as a potent dietary activator of fecundity.

DEDICATION

To all of my teachers at Clemson University, my parents, wife and children

ACKNOWLEDGMENTS

I would like to thank Dr. William Baldwin for giving me the opportunity to work in his laboratory at Clemson University. I would like to thank my committee, Dr. Stephen J Klaine, Dr. Cindy M Lee, Dr. Peter van Den Hurk, and Dr. Robert S Cohen for their continuous input and support throughout my studies. I sincerely express my gratitude to all my teachers at Clemson University. I would like to show my appreciation to Dr. Ellis and Nancy Korn in P&A CAFLS, Clemson University for helping me perform the immunohistochemistry and Dr. Peter van den Hurk for his help with HPLC analysis. I would like to acknowledge my senior colleagues specifically Linda C Mota for her help with qPCR and I want to thank all my colleagues and Sarah Robinson from Dr. Stephen Klaine's lab, who taught me to culture algae. I would like to thank Dr. Patrick Gerard for his statistical advice and the sources that funded my research, George R. MacDonald Fellowship to Gautam Ginjupalli and the National Institutes of Environmental Health Sciences grant R15-ES017321. I would like to thank each and every one who is either directly or indirectly helped me and contributed for improvement of scientific knowledge during my stay in Clemson University.

TABLE OF CONTENTS

Page

TITLE PAGE i

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

CHAPTER

I. INTRODUCTION 1

Daphnia 1
Daphnia Genome 2
Ecological Significance of Daphnia Reproduction 3
Hormonal Regulation of Male Production in Daphnia 5
Juvenile Hormone Analogs 6
Pyriproxyfen 7
Arachidonic Acid 8
Nuclear Receptors 9
Daphnia Nuclear Receptors and HR97 Group of Receptors 11
Specific Aims 12
References 17

II. THE TIME-AND AGE-DEPENDENT EFFECTS OF THE JUVENILE HORMONE ANALOG PESTICIDE PYRIPROXYFEN ON DAPHNIA MAGNA REPRODUCTION 23

Abstract 23
Introduction 24
Materials & methods 27
Daphnia magna culture 27
Male production assays: pyriproxyfen concentration response 28
Male production assays 28
Acute and chronic pyriproxyfen exposure 29
Statistics 30

Results 30
Pyriproxyfen concentration-response 30
Effects of age on male production 33
Acute and chronic pyriproxyfen exposure 34
Discussion 42
References 46

III. ARACHIDONIC ACID ENHANCES REPRODUCTION IN *DAPHNIA MAGNA* AND MITIGATES CHANGES IN SEX RATIOS INDUCED BY PYRIPROXYFEN 51
Abstract 51
Introduction 52
Materials & methods 56
Daphnia magna culture 56
Algal culture 56
Standard acute and chronic toxicity tests 56
Male production assays 57
Influence of different algal diets on reproduction in adult female daphnids 58
Male production assays with Chlorella (low AA diets) 59
Neonate sex determination 59
Statistics 60
Results 60
Fatty acid mediated increases in female production in the presence of pyriproxyfen 60
AA alters pyriproxyfen-mediated neonatal sex ratio 63
Influence of algal diet on reproduction 65
Influence of AA enrichment an AA poor diet on fecundity and neonatal sex ratios 67
Discussion 69
References 75

IV. REPRODUCTIVE AND DEVELOPMENTAL DEFECTS IN *DAPHNIA MAGNA* CAUSED BY IBUPROFEN EXPOSURE ARE DIET-DEPENDENT AND MITIGATED BY ARACHIDONIC ACID 81
Abstract 81
Introduction 82
Materials and Methods 86
Daphnia magna and algae culture 86
Adult reproduction following exposure to ibuprofen 86
Developmental defects induced by ibuprofen 87
Statistics 88
Results 88

Influence of arachidonic acid (AA) rich and poor diets on Daphnia reproduction 88
Developmental defects observed in neonates following ibuprofen exposure 90
Discussion 96
References 99

V. DISCUSSION 103
New findings (Objective-1) 104
New findings (Objective-2) 107
New findings (Objective-3) 109
Purpose of this research 111
References 111

APPENDICES 114
A: Supplementary material for Chapter-2 114
B: Preamble for Chapter-3 117
C: Supplementary material for Chapter-3 121
D: Supplementary material for Chapter-4 122

LIST OF FIGURES

Figure		Page
1.1	Environmental sex determination in Daphnia	4
1.2	Structure of nuclear receptor	9
1.3	Structure of Daphnia juvenile hormone and JHA insecticide……..	14
2.1	Influence of pyriproxyfen on overall fecundity and male production…….	32
2.2	Effects of age on male production	33
2.3	Temporal effects of periodic exposure to pyriproxyfen on reproduction In 3-d old Daphnia magna	35
2.4	Overall effects of temporary exposures to pyriproxyfen on reproduction In 3-d old Daphnia magna	37
2.5	Temporal effects of periodic exposure to pyriproxyfen on reproduction In 10-d old Daphnia magna	39
2.6	Overall effects of temporary exposures to pyriproxyfen on reproduction In 10-d old Daphnia magna	40
3.1	Arachidonic acid alters pyriproxyfen-mediated male/female offspring ratio	62
3.2	Arachidonic acid alters pyriproxyfen-mediated male/female offspring ratio	64
3.3	Influence of algal diets with varying levels of arachidonic acid on Daphnia reproduction	66
3.4	Reproductive effects of supplementing an arachidonic acid poor diet (Chlorella vulgaris) with AA in diet-acclimatized and diet-switched daphnids	68
4.1	Influence of diets rich and poor in AA and arachidonic aid exposures on Daphnia fecundity	89
4.2	Developmental toxicity of ibuprofen observed following	

a C.vulgaris diet 91

4.3 Developmental toxicity of ibuprofen in arachidonic acid (AA) poor and rich diets 93

4.4 ` Percent non-lethal abnormalities produced by ibuprofen exposure 95

CHAPTER ONE

INTRODUCTION

The purpose of this study is to investigate the dietary effects of the polyunsaturated fatty acid, arachidonic acid; better define the non-target effects of the commonly used juvenile hormone analog insecticide, pyriproxyfen, on *Daphnia* reproduction and male production; and reproductive toxicity ibuprofen. Further we evaluated interaction of arachidonic acid (AA), a dietary ω-6 fatty acid with the novel nuclear hormone receptor HR97g and the potential of arachidonic acid in protecting the *Daphnia* from the reproductive toxicity and male producing effects of pyriproxyfen. We also looked into the potential of arachidonic acid in reversing the adverse effects of ibuprofen, an eicosanoid synthesis inhibitor and understand the reproductive and developmental toxicity of ibuprofen in *Daphnia*.

1.1 *Daphnia*

Daphnia, commonly known as water fleas, are branchiopod micro crustaceans in the order *Cladocera* (Dumont and Negrea, 2002), *Daphnia magna* are an aquatic indicator species typically found in freshwater ponds (Carpenter et al., 1987) and are among the most studied organisms in freshwater ecology. They are low to mid-level consumers that filter algae, bacteria, protozoans and detritus for food, and in turn are a source of food for large invertebrates and fish (Gaedke and Straile, 1998; Tessier and Woodruff, 2002). *Daphnia* species are widely used in toxicity testing due to their high sensitivity to a broad range of chemicals, small size, short life-cycles and ease of

culturing in the laboratory (Vandenbrouck et al., 2010). *Daphnia* ecology is well studied accounting for approximately 8% of all available experimental data for aquatic animals (Calzolai et al., 2007). Therefore, they are used to assess the ecological impact of environmental change and a model species for setting up regulatory standards for chemical safety, and monitoring water quality, (Merchant et al., 2008; Colbourne et al., 2011).

1.2 *Daphnia* Genome

Daphnia pulex have a relatively small sequenced genome of 200 Mb (Eads et al., 2007). Recently, the *D. pulex* genome was sequenced (Colbourne et al., 2011) and is considered the environmental genome that is a model to study the interactions of environment with genetics of an organism. *Daphnia pulex* are the first crustaceans fully sequenced, and the first requisite aquatic arthropod sequenced. The *D. magna* genome is currently being sequenced by a consortium of individual *Daphnia* researchers. *Daphnia* are a model organism in aquatic ecology to biomedical sciences (Seda et al., 2011) for studying phenotypic plasticity (Simon et al., 2011), response to oxygen deprivation (Gorr et al., 2004), response to environmental toxicants (USEPA, 1991) and environmental sex determination (Harris et al., 2012). The *Daphnia* genome project demonstrated that daphnids are equipped with the molecular machinery required to adapt to a variety of abiotic and biotic changes in the environment such as presence of predators, scarcity of food, altered photoperiods changes in temperature, overcrowding, and hypoxia (Colbourne et al., 2011).

1.3 Ecological Significance of *Daphnia* Reproduction

Reproduction of *Daphnia* is important in aquatic ecosystems, where *Daphnia* play several roles (Miner et al., 2012). *Daphnia* have short maturation time and typically produce large broods of females through parthenogenesis, building rich populations in a short time span (Anderson and Jenkins, 1942). Under optimal conditions that foster growth and reproduction, *Daphnia* release a brood every two to three days until the death of the adult (Ebert, 2005). Therefore, a healthy *Daphnia* population that produces rich populations in a short span is crucial for subsistence of an ecosystem, where they are a mid-level consumer and food source for small fish (Gaedke and Straile). As a primary consumer of algae, *Daphnia* contribute to keeping algal blooms and the problems associated with algal blooms under check (Miner et al., 2012). In addition, *Daphnia* are considered important in trophic transfer of nutrients in aquatic food webs as they are eaten by fish and other larger invertebrates (Dodson and Hanazato, 1995). A healthy and actively reproducing population of *Daphnia* is highly desirable and any influence that disrupts the parthenogenic mode of reproduction might adversely affect the entire ecosystem.

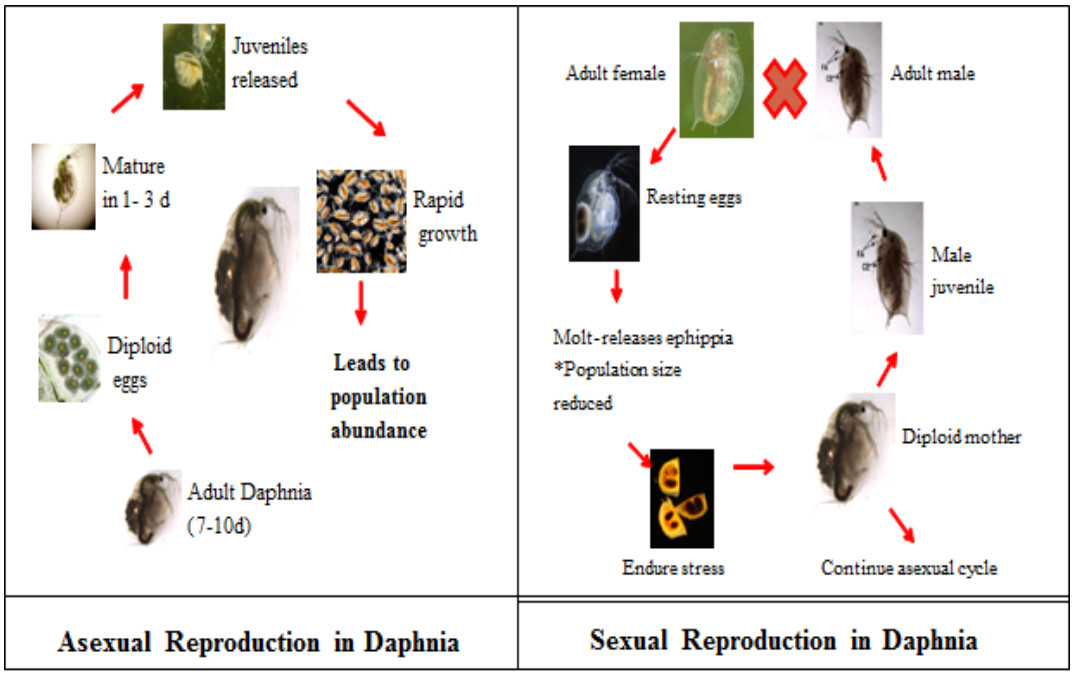


Figure.1.1 Environmental Sex Determination in *Daphnia*: Asexual and sexual reproduction in *Daphnia* species. Under ideal conditions, the *Daphnia* population is primarily parthenogenic females, but during specific stressors such as overcrowding, changes in food allocation, and others; *Daphnia* produce males for sexual reproduction.

Most *Daphnia* strains (both in the laboratory and in ponds) will switch to sexual reproduction under stressful conditions and produce males. Adult female that sense the harsh conditions produce haploid males. Males produced under stress copulate with a receptive female in the vicinity, which produces diploid diapause eggs protected by ephippia that survive harsh environmental conditions and continuation of asexual reproduction under more favorable conditions (Olmstead and LeBlanc, 2002). Several environmental cues such as temperature, photoperiod, and overcrowding (Deng, 1997; Hobæk and Larsson, 1990) induce male production in *Daphnia*. Overcrowding is the primary factor responsible for initiation of sexual reproduction in daphnids. But, some scientists believe that perturbations in at least two environmental factors are necessary for

sexual reproduction. The genes associated with sexual reproduction (Stillman et al., 2008) and the receptor for methyl farnesoate responsible for male production have been recently investigated (Miyakawa et al., 2013). The receptor is a Per-Arnt-Sim (PAS) type transcription factor. Male production was thought to be associated with costs in terms of energy expenditure producing males, large body of empirical and theoretical work on resolving the paradox and cost of sex exists. However, male production in *Daphnia pulex* was observed to be associated with costs, only under resource limited environment, especially when sexual and asexual lineages are raised separately (Wolinska and Lively, 2008). However, we have not considered the costs associated with male production in terms of the effects on fecundity or survival.

1.4 Hormonal Regulation of Male Production in *Daphnia*

Methyl farnesoate, which is the unepoxidated form of juvenile hormone iii is actively synthesized by the female crustacean mandibular organ during vitellogenesis (Laufer, 1992). It is the major signaling molecule involved in regulating larval development (Yamamoto et al., 1997), juvenile morphological characters (Laufer and Biggers, 2001) and embryonic and juvenile development similar to insects (Olmstead and LeBlanc, 2003). Methyl farnesoate is proved as the key sex determinant in the branchiopod crustacean *Daphnia magna* and induces male production in early stages of juvenile development (Olmstead and LeBlanc, 2002). Timing is crucial in male production as exposure must occur during a 12-h period in ovarian oocyte maturation just prior to the transfer of the eggs to the brood chamber (Olmstead and LeBlanc, 2002).

Therefore, it may take as many as three broods after pyriproxyfen or methyl farnesoate exposure before a significant number of males are produced (Kishi et al., 2005). Interestingly, hypoxia and male production may be associated as methyl farnesoate induces Hb2 (Gorr et al., 2004), a key gene in response to hypoxia. A key question is whether increased Hb synthesis under hypoxia and overcrowding turns on methyl farnesoate signaling cascade or overcrowding, independent of Hb causes male production through the release of hormonal factors.

1.5 Juvenile Hormone Analogs

Pest management has great economic impact on agricultural production, as it is directly linked to the yield. There is a never-ending search for developing new pesticides or insecticides that are safer to human and the environment. Therefore, juvenile hormone analogs (JHAs) that possess both of these properties without compromising their efficacy are among the preferred insecticides. Unlike many other insecticides that are in use, the JHAs do not kill the insects directly. Instead, they act as insect growth regulators (IGRs) by interfering with the insect endocrine system that in turn prevents larval maturation, metamorphosis and ultimately leads to the death of larvae. Fenoxycarb, methoprene, kinoprene and pyriproxyfen are the commonly used JHAs with varying levels of efficacy. Among the commonly used JHAs pyriproxyfen is of special interest to us because of the non-target effects on *Daphnia.*

1.6 Pyriproxyfen

Pyriproxyfen, (2-[1-methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine) is used in household, agricultural, and horticultural applications to control many insect species, including the common housefly (*Musca domestica*), mosquitoes, imported red fire ants (*Solenopsis invicta*), and silverleaf whitefly (*Bemisia argentifolii*) (Sullivan and Goh, 2008).

Pyriproxyfen has a moderate vapor pressure of 1.0×10^{-7} mmHg at 20^0C with a slight tendency to vaporize from aquatic sources and enter the atmosphere. It has a solubility is 0.367 ppm and stable in aquatic environments with a photolytic half-life of 3.72-6.23 d in aquatic environment and a filed dissipation half-life of 3.5 to 16.5 d. Its photolytic half-life is approximately 6.8-8.5 d in soil under artificial light (Sullivan, 2000). Pyriproxyfen has an aerobic soil metabolism half-life of 12.4 d, aquatic aerobic metabolic half-life of 23.1 d and an anaerobic aquatic metabolism half-life of 346.5 d (Sullivan, 2000). The physicochemical properties of pyriproxyfen indicate its stability in the aquatic environment with minimum mobility across different strata. Pyriproxyfen is relatively safe for mammals with an oral LD50 > 5000 mg/Kg, a dermal LD50 > 2000 and an acute inhalation LD50 > 1000 mg/Kg in rats (Invest and Lucas, 2008).

The relative stability and slower movement of pyriproxyfen in the environment contributes to the long acting potential of pyriproxyfen that make it one of the preferred insecticides used for pest control in pets. Several of the over the counter topical applications for the control of flea and tick infestation in dogs and cats contain pyriproxyfen (Michael, 2005). Pyriproxyfen protects cats from recurrence of flea

infestation for about six months (Maynard et al., 2001) although it does not kill adult fleas and ticks. This is because pyriproxyfen is a potent juvenile hormone agonist and endocrine disruptor (Sullivan and Goh, 2008) that inhibits larval development and maturation (Meola et al., 2000) as a juvenile hormone iii mimic (Wang et al., 2005). Therefore, pyriproxyfen preferentially perturbs juvenile development and hatching success of flea and tick eggs.

1.7 Arachidonic Acid

The dietary ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) is important in arthropod physiology. PUFAs, especially highly unsaturated fatty acids, contribute to the fluidity of cell membranes in fish and cladocerans and help these organisms to withstand cold temperatures (Michael et al., 1997). Fish fed on zooplankton rich in PUFAs exhibit higher growth rates and fecundity (Verreth et al., 1994) and zooplankton grazing on PUFA rich phytoplankton exhibit higher growth rates and fecundity (Michael et al., 1997). Mechanisms associated with *Daphnia* responses to environmental cues are not clearly known, however, their efficiency in allocating limited dietary resources is crucial to survival and reproductive preferences (Meester et al., 2011). *Daphnia* preferentially accumulate the dietary PUFAS, AA and eicosapentaenoic acid (EPA), and much of it is allocated to the ovary in the late stages of oocyte maturation (Ahlgren et al., 1990; Goulden and Place, 1993; Bec et al., 2003; Wacker and Martin-Creuzburg, 2007; Taipale et al., 2011).

PUFAs are important as precursors of prostaglandins through the cyclooxygenase pathway from AA (Needleman et al., 1986). We hypothesized that dietary PUFAs are

retained in the daphnid ovary because they are crucial in reproduction. AA also inhibits the novel nuclear receptor group, HR97g, found primarily in adult ovary and gastrointestinal tract and weakly activated by the male-producing JHA, pyriproxyfen (Ginjupalli, 2011). Therefore, we considered a diet sufficient in PUFAs and specifically AA may help repress male production.

AA is a precursor to eicosanoids that are associated with several important physiological functions (Stanley, 2006; Büyükgüzel et al., 2011) such as immune function in insects (Miller et al., 1994) and crustaceans (Heckmann et al., 2008). Eicosanoids are important in immune functions, reproduction and ion transport in mammals (Hayashi et al., 2008). Accumulation of AA in the ovaries of marine shrimp *Penaeus semisulcatus* is associated with oocyte maturation (Ravid et al., 1999). The prostaglandin, PGE_2, a metabolite of AA from the cyclooxygenase pathway is associated with egg laying behavior in insects (Stanleysamuelson and Loher, 1986), and $PGF_{2\alpha}$ is responsible for enhanced sperm motility and ovulation in mammals (Chang et al., 1997). Therefore, accumulation of AA in the ovary is important as AA may directly influence reproduction or act through the production of eicosanoids to function as signaling molecules responsible for egg maturation or release.

1.8 Nuclear receptors

Figure.1.2 Structure of Nuclear Receptor

Nuclear receptors comprise a super family of transcription factors that are both ligand dependent and independent. Study of the nuclear receptors is important in understanding how they regulate the transcription of target genes associated with development, reproduction, metabolism and homeostasis of organisms. A typical nuclear receptor contains five distinct functional domains named as A/B, C, D, E and F (Evans, 2005). Each of the five domains performs distinct functions in transcriptional regulation of the target genes. The variable N-terminal A/B domain is highly variable and consists of at least one transactivation region (AF-1) involved in activating the basal transcriptional complex and several other transactivation domains (ADs) (Robinson-Rechavi et al., 2003). They recruit coactivators and release of repressors following the binding of a suitable ligand at a moderately conserved E domain known as Ligand Binding Domain (LBD). The LBD also consists of the Activation Function-2 domain (AF-2), which when not bound by ligand, helps repress transcription. The highly conserved C domain is the binding domain (DBD), and is responsible for dimerizing with another nuclear receptor partner and then binding to the DNA at enhancer elements. The DBD contains two zinc finger motifs and binds the DNA at a place known as the response element ahead of the promoter region of the target gene. Between the C domain and the E domain lies the less conserved D domain that acts as a hinge and contains the Nuclear Localization Signal (NLS) is concerned with translocation of nuclear receptor (Robinson-Rechavi et al., 2003). The C-terminal F domain is highly variable and may not be found in all of the nuclear receptors (Blumberg, 1998; Weatherman et al., 1999). Its

exact role is not known, but the F domain may be involved in repressing nuclear receptor activity in some receptors.

Based on the mode of activation and dimerization properties the nuclear receptors are classified as Type I or Type II receptors. Type I receptors that bind to palindromic repeats in a homodimers following activation by a ligand, but otherwise remain in an inactive state in the cytosol, often bound to a cytosolic repressor such as heat shock proteins (HSPs) or cytosolic retention proteins (MCkenna et al., 1999; Hernandez et al., 2009). On the other hand, the type II, can bind to DNA without any ligand dependent activation at direct repeat sequences at the response element. These receptors are constitutively active and exhibit greater promiscuity in dimerization patterns such as heterodimerization with retinoid-X-receptor (RXR), and found to repress transcription (McKenna et al., 1999).

The typical five domain structure and the usual ligand dependent activation of nuclear receptors is not is a general rule, because of few exceptions to this general rule. For example the Constitutive Androstane Receptor (CAR) has a three domain structure instead of five domains (Suino et al., 2004), and several receptors show constitutive activity independent of ligand activation.

1.9 *Daphnia* Nuclear Receptors and HR97 Group of Receptors

The *Daphnia pulex* genome sequencing project identified 25 nuclear receptors, including a novel group of receptors called the HR97 group of receptors with three members, HR97a, HR97b, and HR97g, that are classified into a distinct group designated

as NR1L with unknown functions. They share structural similarities with HR96, a member of the NR1J subfamily related to the metabolic and xenobiotic sensing nuclear receptors in vertebrates. The HR97 group of receptors also show some relatedness to the NR0A group (knirps) that lack ligand binding domains and are involved in development (Thomson et al., 2009). An unknown protein with a size of 55kDa was found to bind the promoter element of Hb2 after methyl farnesoate exposure (Gorr et al., 2004). Interestingly, the size of HR97g receptor matches with that of the unknown protein of 55kDa found on Hb2 promoter element and (RXR is only 46kDa), therefore we hypothesized that HR97g may be a key nuclear receptor in male production.

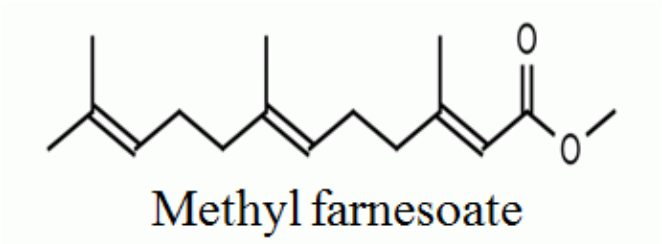
1.10 Specific Aims

Daphnia magna reproduce by parthenogenesis under favorable conditions and switch to sexual reproduction under specific adverse conditions. Mechanisms underlying environmental sex determination are complex. The molecular pathways that sense the stressors and induce sexual cycles under stress are not fully understood. In this study we will investigated some of the male production dynamics of pyriproxyfen more specifically in a time concentration and age dependent fashion in *Daphnia magna* and the ability of arachidonic acid a dietary ω-6 fatty acid in ameliorating the adverse impacts of exposure to juvenile hormone analogs.

Objective 1:

Determine the reproductive, male production, and temporal effects of pyriproxyfen on *Daphnia magna*.

The sexual reproductive cycle in daphnids requires the production of male animals that mate with adult females leading to the production of usually two resting eggs embedded in a tough protective case called an ephippium, a strategy adapted by *Daphnia* to endure adverse stressful conditions. We will test the effects of pyriproxyfen on fecundity and male production in *D.magna* under different conditions, including dose-dependent, age-dependent, and the pulsatile effects of JHA exposure on fecundity and male production. Sexual reproduction enables a population of *Daphnia* to survive adverse conditions. However, the induction of sexual reproduction also significantly reduces fecundity, which can impact the ecosystem as algal growth may cause eutrophic conditions, and predators may suffer lack food. Therefore, the chemically-induced onset of sexual reproduction can have significant adverse effects on population dynamics and the ecosystem. Research findings from this study published as Ginjupalli, G.K., Baldwin, W.S, 2013. The time-and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on *Daphnia magna* reproduction. Chemosphere. 92, 1260-06 is presented as chapter two.



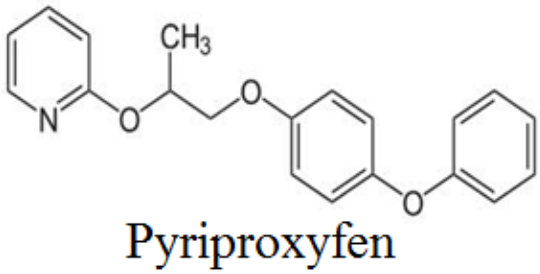


Figure.1.3 Structure of *Daphnia* Juvenile hormone and JHA insecticide

Objective 2:

Test whether the HR97g inhibitor, arachidonic acid, blocks male production.

Arachidonic acid is a HR97g blocker in vitro at 10μM. Assays using arachidonic acid as a pharmacological inhibitor of HR97g were performed to test whether arachidonic acid treatment in vivo could block male production. We determined the acute and chronic toxicity effects of arachidonic acid in a 21-day life cycle test. Pyriproxyfen is known to induce male production in the non-target crustacean *Daphnia* at environmentally relevant concentrations of approximately 330 pM. Interestingly, the novel *Daphnia* nuclear hormone receptor HR97g expressed in the reproductive tissues of *Daphnia* is inhibited by arachidonic acid, a ω-6 dietary fatty acid in vitro. Furthermore, AA is accumulated in the

ovaries along with EPA from diet. Therefore, we hypothesized that interactions of AA and HR97g are important in reproduction and may be important in regulating the neonatal sex ratios as a means to repress environmental sex determination in *Daphnia* as the current diet is good or excellent. We conducted reproduction assays using AA and pyriproxyfen to determine the potential of AA as an inhibitor of the HR97g receptor and concurrent reproductive toxicity of pyriproxyfen such as fecundity, male production and overall reproduction in the absence and presence of pyriproxyfen. Research findings of this study are presented as preamble for chapter in the Appendx-3 and the results from arachidonic acid interactions with fecundity and male production in presence of pyriproxyfen are submitted for publication to Ecotoxicology & Environmental were included in chapter three.

Objective 3:

Determine the adverse developmental effects of the eicosanoid synthesis inhibitor ibuprofen in *Daphnia.*

Arachidonic acid is accumulated in the ovaries of the *Daphnia*. We and several others hypothesized that it is important in reproduction. In addition metabolites of AA are implicated in several physiological roles such as in inflammation, immunity and signaling process. PGE2 a metabolite of AA is known to influence the egg laying behavior in insects. Fish and shrimp that were fed on AA rich diets were observed to show higher growth rates and spawning. However, the role of AA in *Daphnia* reproduction is not precisely known. Therefore, we investigated whether the beneficial effects of AA are in

part mediated by its metabolites. We decided to use ibuprofen as an inhibitor of AA and its metabolite synthesis. Acute and chronic toxicity assays were conducted to determine the EC50 value of ibuprofen. Further, *Daphnia* were raised on diets that are moderately rich and poor in AA for several generations to modulate accumulation of AA. *Daphnia* were treated with ibuprofen at several different concentrations or AA alone or in combinations of AA and ibuprofen and examine the influence on *Daphnia* reproduction. Further, the ability of AA supplemented through diet in recovering the reproduction under different dietary conditions in presence of ibuprofen was evaluated. Ibuprofen exposures are aimed at inhibiting the endogenous AA synthesis. This artificial inhibition of AA synthesis was hypothesized to cause adverse effects on *Daphnia* reproduction, if AA was important for *Daphnia* reproduction. The beneficial effects of dietary AA supplementation in these *Daphnia* in reproduction recovery provided evidence for the purported role of AA in *Daphnia* reproduction. In addition, the transgenerational influence of feeding AA rich diet in reversing the adverse reproductive effects of ibuprofen were determined by acclimatizing the neonates to different dietary conditions without fish food. Neonates from first and second generations raised on *P. subcapitata* without fish food were treated with ibuprofen or AA or in combinations of IB+AA to determine whether *Daphnia* use dietary or the supplemented AA to reverse the reproductive toxicity of ibuprofen. The research findings from this study were included in chapter four. The manuscript will be submitted soon.

1.11 References

Ahlgren, G., Lundstedt, L., Brett, M., Forsberg, C., 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. J. Plankton. Res. 12, 809-818

Anderson, B.G., Jenkins, J.C., 1942. A time study of events in the life span of *Daphnia Magna*. Biol. Bull. 83, 260-272.

Bec, A., Desvilettes, C., Vera, A., Lemarchand, C., Fontvieille, D., Bourdier, G., 2003. Nutritional quality of a freshwater heterotrophic flagellate: Trophic upgrading of its microalgal diet for *Daphnia hyalina*. Aquat. Microb. Ecol. 32, 203-207.

Blumberg, B.a.E., RM, 1998. Orphan nuclear receptors—new ligands and new possibilities. Genes Dev. 12, 3149-3155.

Büyükgüzel, E., Tunaz, H., Stanley, D., Büyükgüzel, K., 2011. The influence of chronic eicosanoid biosynthesis inhibition on life history of the greater waxmoth, *Galleria mellonella* and its ectoparasitoid, *Bracon hebetor*. J. Insect Physiol. 57, 501-507.

Calzolai, L., Ansorge, W., Calabrese, E., Denslow, N., Part, P., Lettieri, T., 2007. Transcriptomics and proteomics. Applications to ecotoxicology. Comp. Biochem. Physiol. Part D Genomics Proteomics. 2, 245-249.

Carpenter, S., Kitchell, J., Hodgson, J., Cochran, P., Elser, J., Elser, M., Lodge, D., Kretchmer, D., He, X., Von, E., 1987. Regulation of lake primary productivity by food web structure. Ecology. 68, 1863-1876.

Chang, K.J., Kim, J.W., Im, W.B., Kang, H.M., Kwon, H.B., 1997. Differential effects of gonadotropin and orthovanadate on oocyte maturation, ovulation, and prostaglandin synthesis by *Rana* ovarian follicles in vitro. J. Exp. Zool. 277, 155-165.

Colbourne, J.K., Pfrender, M.E., Gilbert, D., Thomas, W.K., Tucker, A., Oakley, T.H., Tokishita, S., Aerts, A., Arnold, G.J., Basu, M.K., Bauer, D.J., Caceres, C.E., Carmel, L., Casola, C., Choi, J.H., Detter, J.C., Dong, Q.F., Dusheyko, S., Eads, B.D., Frohlich, T., Geiler-Samerotte, K.A., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E.V., Kultz, D., Laforsch, C., Lindquist, E., Lopez, J., Manak, J.R., Muller, J., Pangilinan, J., Patwardhan, R.P., Pitluck, S., Pritham, E.J., Rechtsteiner, A., Rho, M., Rogozin, I.B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, Z.J., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y.I., Yamagata, H., Yamada, T., Ye, Y.Z., Shaw, J.R., Andrews, J., Crease, T.J., Tang, H.X., Lucas, S.M., Robertson, H.M., Bork, P., Koonin, E.V., Zdobnov, E.M., Grigoriev, I.V.,

Lynch, M., Boore, J.L., 2011. The ecoresponsive genome of *Daphnia pulex*. Science. 331, 555-561.

Deng, H., 1997. Photoperiodic response of sexual reproduction in the *Daphnia Pulex* group is reversed in two distinct habitats. Limnol. Oceanogr. 42, 609-611.

Dodson, S.I., Hanazato, T., 1995. Commentary on effects of anthropogenic and natural organic-chemicals on development, swimming behavior, and reproduction of *Daphnia*, a key member of aquatic ecosystems. Environ. Health. Perspect. 103, 7-11.

Dumont, H.J., Negrea, S., 2002. Introduction to the class branchiopoda. NHBS, Pub: Backhuys, University of California.

Eads, B., Andrews, J., Colbourne, J., 2007. Ecological genomics in *Daphnia*: stress responses and environmental sex determination. Heredity. 100, 184-190.

Ebert, D., 2005. Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*. National library of medicine (U.S), National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books

Evans, R.M., 2005. The nuclear receptor superfamily: A rosetta stone for physiology. Mol. Endocrinol. 19, 1429-1438.

Gaedke, U., Straile, D., 1998. Daphnids: Keystone species for the pelagic food web structure and energy flow - A body size-related analysis linking seasonal changes at the population and ecosystem levels. Advance. Limnol. 53, 587-610.

Gorr, T.A., Cahn, J.D., Yamagata, H., Bunn, H.F., 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-induced factor dependent. J. Biol. Chem. 279, 36038-36047.

Goulden, C.E., Place, A.R., 1993. Lipid accumulation and allocation in daphnid cladocera. Bull. Marine Sci. 53, 106-114.

Harris, K., Bartlett, N., Lloyd, V., 2012. *Daphnia* as an emerging epigenetic model organism. Genet. Res. Int. 2012:8.

Hayashi, Y., Heckmann, L.H., Callaghan, A., Sibly, R.M., 2008. Reproduction recovery of the crustacean *Daphnia magna* after chronic exposure to ibuprofen. Ecotoxicol. 17, 246-251.

Heckmann, L.H., Sibly, R.M., Timmermans, M., Callaghan, A., 2008. Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. Front. Zool. 5:11.

Hernandez, J.P., Mota, L.C., Huang, W., Moore, D.D., Baldwin, W.S., 2009. Sexually dimorphic regulation and induction of P450s by the constitutive androstane receptor (CAR). Toxicology. 256, 53-64.

Hobæk, A., Larsson, P., 1990. Sex determination in *Daphnia Magna*. Ecology. 71, 2255-2268.

Invest, J., Lucas, J., 2008. Pyriproxyfen as a mosquito larvicide. Robinson, W., Bajomi, D. (Eds.). Sixth International Conference on Urban Pests. OOK press,Kft, Hungary, 239-245.

Karimullina, E., Li, Y., Ginjupalli, G.K., Baldwin, W.S., 2012. *Daphnia* HR96 is a promiscuous xenobiotic and endobiotic nuclear receptor. Aquat. Toxicol. 116, 69-78.

Kishi, R., Sata, F., Katakura, Y., Wang, R.-S., Nakajima, T., 2005. Effects of pregnancy, age and sex in the metabolism of styrene in rat liver in relation to the regulation of cytochrome P450 enzymes. J. Occup. Health. 47, 49-55.

Laufer, H., Method for increasing crustacean larval production. U.S. Patient, 5161481 A, Sept 28, 1992.

Laufer, H., Biggers, W.J., 2001. Unifying concepts learned from methyl farnesoate for invertebrate reproduction and post-embryonic development. Amer. Zool. 41, 442-457.

Maynard, L., Houffschmitt, P., Lebreux, B., 2001. Field efficacy of a 10 per cent pyriproxyfen spot-on for the prevention of flea infestations on cats. J. Small Anim. Pract. 42, 491-494.

McKenna, N., Lanz, R., O'malley, B., 1999. Nuclear receptor coregulators: Cellular and molecular biology. Endocrine Reviews 20, 321–344.

Meester, L., Doorslaer, W., Geerts, A., Orsini, L., Stoks, R., 2011. Thermal genetic adaptation in the water flea *daphnia* and its impact: An evolving metacommunity approach. Integr. Comp. Biol. 51, 703–718

Meola, R., Meier, K., Dean, S., Bhaskaran, G., 2000. Effect of pyriproxyfen in the blood diet of cat fleas on adult survival, egg viability, and larval development. J. Med. Entomol. 37, 503-506.

Merchant, D., Ertl, R.L., Rennard, S.I., Stanley, D.W., Miller, J.S., 2008. Eicosanoids mediate insect hemocyte migration. J. Insect Physiol. 54, 215-221.

Michael, B., Dorthe, M., Muller, N., 1997. The role of highly unsaturated fatty acids in aquatic food web processes. Freshw. Biol. 38, 483–499.

Michael, K.R., 2005. Advances in the control of *Ctenocephalides felis* (cat flea) on cats and dogs. Trends in Parasitol. 21, 232-236.

Miller, J.S., Nguyen, T., Stanleysamuelson, D.W., 1994. Eicosanoids mediate insect nodulation responses to bacterial-infections. Proceedings of the Nat Acad Sci of the United States of America. 91, 12418-12422.

Miner, B.E., De Meester, L., Pfrender, M.E., Lampert, W., Hairston, N.G., 2012. Linking genes to communities and ecosystems: *Daphnia* as an ecogenomic model. Proceedings of the Royal Society B-Biol. Sci. 279, 1873-1882.

Miyakawa, H., Toyota, K., Hirakawa, I., Ogino, Y., Miyagawa, S., Oda, S., Tatarazako, N., Miura, T., Colbourne, J.K., Iguchi, T., 2013. A mutation in the receptor methoprene-tolerant alters juvenile hormone response in insects and crustaceans. Nat. Commun. 4,1856.

Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., Lefkowith, J.B., 1986. Arachidonic-acid metabolism. Annu. Rev. Biochem. 55, 69-102.

Olmstead, A.W., LeBlanc, G.A., 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. J. Exp. Zool. 293, 736-739.

Olmstead, A.W., LeBlanc, G.A., 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environ. Health Perspect. 111, 919-924.

Ravid, T., Tietz, A., Khayat, M., Boehm, E., Michelis, R., Lubzens, E., 1999. Lipid accumulation in the ovaries of a marine shrimp *Penaeus semisulcatus* (De Haan). J. Exp. Biol. 202, 1819-1829.

Robinson-Rechavi, M., Garcia, H., Laudet, V., 2003. The nuclear receptor superfamily. J. Cell Sci. 116, 585-586.

Seda, J., Petrusek, A., Sehnal, F., 2011. *Daphnia* as a model organism in limnology and aquatic biology: Some aspects of its reproduction and development. J. Lymnol. 70, 336-336.

Simon, J.C., Pfrender, M.E., Tollrian, R., Tagu, D., Colbourne, J.K., 2011. Genomics of environmentally induced phenotypes in 2 extremely plastic arthropods. J. Heredity. 102, 512-525.

Stanley, D., 2006. Prostaglandins and other eicosanoids in insects: Biological significance. Ann. Rev. Entomol. 25-44.

Stanleysamuelson, D.W., Loher, W., 1986. Prostaglandins in insect reproduction. Annals Entomol. Soci. Amer. 79, 841-853.

Stillman, J.H., Colbourne, J.K., Lee, C.E., Patel, N.H., Phillips, M.R., Towle, D W, Eads, B.D., Gelembuik, G.W., Henry, R.P., Johnson, E.A., Pfrender, M.E., Terwilliger, N.B., 2008. Recent advances in crustacean genomics. Integr. Comp. Biol. 48, 852-868.

Suino, K., Peng, L., Reynolds, R., Li, Y., Cha, J., Repa, J., Kliewer, S., Xu, H., 2004 The nuclear xenobiotic receptor CAR: Structural determinants of constitutive activation and heterodimerization. Mol. Cell. 6, 893-905.

Sullivan, J., Goh, K., 2008. Environmental fate and properties of pyriproxyfen. J. Pest Sci. 33, 339-350.

Taipale, S.J., Kainz, M.J., Brett, M.T., 2011. Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids in *Daphnia*. Oikos (Epub ahead of print: doi: 10.1111/j.1600-0706.2011.19415.x).

Tessier, A., Woodruff, P., 2002. Trading off the ability to exploit rich versus poor food quality. Ecol. Let. 5, 685-692.

Thomson, S.A., Baldwin, W.S., Wang, Y.H., Kwon, G., LeBlanc, G.A., 2009. Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. BMC Genomics. 10, 500.

USEPA,(United States Environmental Protection Agency) 1991. Methods For Measuring The acute toxicity of effluents and receiving waters to freshwater and marine organisms. (Ed) Weber, C.I., Report number: EPA-600/4-90-02. Edn: 4. Office of Research and development, Washington, DC.

Vandenbrouck, T., Jones, O., Dom, N., Griffin, J., De Coen, W., 2010. Mixtures of similarly acting compounds in *Daphnia magna*: From gene to metabolite and beyond. Environ. Int. 36, 254-268.

Verreth, J., Coppoolse, J., Segner, H., 1994. The effect of low HUFA- and high HUFA-enriched *Artemia*, fed at different feeding levels, on growth, survival, tissue fatty acids and liver histology of *Clarias gariepinus* larvae. Aquaculture. 126, 137-150.

Wacker, A., Martin-Creuzburg, D., 2007. Allocation of essential lipids in *Daphnia magna* during exposure to poor food quality. Funct. Ecol. 21, 738-747.

Wang, H.Y., Olmstead, A.W., Li, H., LeBlanc, G.A., 2005. The screening of chemicals for juvenoid-related endocrine activity using the water flea *Daphnia magna*. Aquat. Toxicol. 74, 193-204.

Weatherman, R., Fletterick, R., Scanlan, T., 1999. Nuclear-receptor ligands and ligand-binding domains. Annu. Rev. Biochem. 68, 559-581

Wolinska, J., Lively, C.M., 2008. The cost of males in *Daphnia pulex*. Oikos 117, 1637-1646.

Yamamoto, H., Okino, T., Yoshimura, E., Tachibana, A., Shimizu, K., Fusetani, N., 1997. Methyl farnesoate induces larval metamorphosis of the barnacle, *Balanus amphitrite* via protein kinase C activation. J. Exp. Zool. 278, 349-355.

CHAPTER TWO

THE TIME-AND AGE-DEPENDENT EFFECTS OF THE JUVENILE HORMONE ANALOG PESTICIDE, PYRIPROXYFEN ON DAPHNIA MAGNA REPRODUCTION

2.1 ABSTRACT

Pyriproxyfen is an insecticidal juvenile hormone analog that perturbs insect and tick development. Pyriproxyfen also alters parthenogenic reproduction in non-target cladoceran species as it induces male production that can lead to a decrease in fecundity, a reduction in population density, and subsequent ecological effects. In this study, we investigate the impacts of pyriproxyfen on *Daphnia magna* reproduction using a series of male production screening assays. These assays demonstrate that pyriproxyfen increases male production in a concentration-dependent fashion with an EC50 of 156 pM (50.24 ng L^{-1}); a concentration considered environmentally relevant. Furthermore, pyriproxyfen decreases overall fecundity at all ages tested (7, 14, 21-d old female parthenogenic daphnids). Juvenile (3-d old) and reproductively mature (10-d old) female daphnids were also exposed to 155 pM pyriproxyfen for 2–12 d and reproduction measured for 16 d to compare the effects of short-term and prolonged exposures, and determine the potential for recovery. Results indicate that longer pyriproxyfen exposures (8–12 d) extend male production and decrease reproduction; however, daphnids exposed for only 2–4 d recover and produce a relatively normal abundance of neonates. In addition, juvenile daphnids are also very sensitive to pyriproxyfen, but the primary effect on juvenile daphnids is reduced reproduction and protracted development not male production. Taken together, continued use of pyriproxyfen around water bodies needs due caution because of its potential

adverse effects with significant developmental delays and male production compounded by prolonged exposure.

2.2 Introduction

Pyriproxyfen, (2-[1-methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine) is used in household, agricultural, and horticultural applications to control many insect species, including the common housefly (*Musca domestica*), mosquitoes, imported red fire ants (*Solenopsis invicta*), and silverleaf whitefly (*Bemisia argentifolii*) (Sullivan and Goh, 2008). In addition, several over the counter topical applications for the control of flea and tick infestation in dogs and cats contain pyriproxyfen (Michael, 2005). Pyriproxyfen protects cats from recurrence of flea infestation for about six months (Maynard et al., 2001) although it does not kill adult fleas and ticks. This is because pyriproxyfen is a potent juvenile hormone agonist and endocrine disruptor (Sullivan and Goh, 2008) that inhibits larval development and maturation (Meola et al., 2000) as a juvenile hormone iii mimic (Wang et al., 2005). Therefore, pyriproxyfen preferentially perturbs juvenile development and hatching success of flea and tick eggs.

Pyriproxyfen is relatively stable in the environment (Katagi and Takahashi, 1994), and is active in artificial *Aedes aegypti* breeding sites for five months (Darriet et al., 2010). Pyriproxyfen is also relatively lipophilic as it has an octanol/water partitioning coefficient of K_{ow} $10^{5.6}$ and a bioconcentration factor (BCF) of approximately 1500 in fish (Steginsky et al., 1994). Some models predict that pyriproxyfen may have a greater BCF in aquatic organisms with lower metabolic capacity based on its K_{ow} (Meylan et al.,

1999; Sullivan and Goh, 2008). The maximum aquatic pyriproxyfen concentrations in surface waters are estimated to be about 290 pM (93 ng L^{-1}) based on a New York State risk assessment (Serafini, 2001), but may approach 1245 pM (400 ng L^{-1}) 24 h post-spray based on field tests (Schaefer and Miura, 1990). Consequently, there is concern that run-off from commercial and household applications could bioaccumulate and affect non-target species such as crustaceans (Tuberty and McKenney, 2005), especially parthenogenically reproducing cladocerans such as *Daphnia* (Olmstead and LeBlanc, 2003).

Daphnia are non-target, branchiopod crustaceans found in freshwater ponds all over the world. They are mid-level consumers and filter feeders that primarily eat algae and are preyed upon by larger arthropods and fish (Carpenter et al., 1987). There is a large body of research indicating their role in aquatic ecology (Ebert, 2005). *Daphnia magna* is commonly used in the assessment of environmental toxicants (Dang et al., 2012; OECD, 2012), which is due to its amenability to laboratory culture and parthenogenic reproduction. Under ideal conditions, *D. magna* females can make clones of themselves with broods up to 50 neonates every 2–3 d (OECD, 2012).

However, under poor conditions such as overcrowding (Smith et al., 2009), poor food quality (Koch et al., 2009), shortage of food (Kleiven et al., 1992) or reduced photoperiod (Deng and Lynch, 1996), females produce males required for sexual reproduction. Sexual reproduction leads to the production and release of ephippia containing resting eggs that can survive difficult conditions including desiccation (Ebert, 2005). Most juvenile hormone analogs, including the endogenous crustacean juvenoid,

methyl farnesoate, induce male production in *D. magna* (Olmstead and LeBlanc, 2003; Tatarazako et al., 2003) and other cladoceran species (Oda et al., 2005b).

Pyriproxyfen is one of the most efficacious of these juvenile hormone analogs, and consequently it is included in the United States Environmental Protection Agency (USEPA) draft list for Tier 1 screening of endocrine disrupting chemicals (USEPA, 2007). Pyriproxyfen has a male production EC50 of about 170–310 pM in *D. magna* (55–100 ng L^{-1}) (Olmstead and LeBlanc, 2003; Matsumoto et al., 2008). Therefore, environmentally relevant concentrations could increase male production in *Daphnia* and other cladocerans. Increases in male production in *Daphnia* species decreases the output of parthenogenic females, reduces population growth rate, and ultimately population abundance (Olmstead and LeBlanc, 2003). Daphnids play a crucial role as keystone species in aquatic ecosystems as primary consumers of algae and a source of food for larger invertebrates and fish (Gaedke and Straile, 1998). Perturbations in *Daphnia* abundance could lead to environmental problems such as algal blooms, reduced fish populations, and overall disruption of the aquatic ecosystem. Therefore, pyriproxyfen must be sprayed with caution around aquatic environments (Serafini, 2001; Sullivan and Goh, 2008).

The ability of an organism to recover is crucial to ecosystem health, and it has been proposed that early stage exposure to endocrine disruptors may permanently damage endocrine systems and contribute to reproductive and carcinogenic effects in adults (Nichols et al., 2011; Chen et al., 2012). If individuals are unable to recover from toxic insults than recovery of ecosystems may be perturbed. It has been suggested that

male production assays could be used as tier 2 screens for the USEPA's Endocrine Disruptor Screening Program (Wang et al., 2005), and this screen and other screens if modified could be used to assess the ability of organisms to recover from exposure to endocrine disruptors. Therefore, the reproductive and male producing effects of acute and chronic exposures to pyriproxyfen were examined in juvenile and adult *D. magna*.

We investigated the reproductive toxicity and male producing effects of pyriproxyfen to *D. magna*, and tested whether acute (2–4 d) and chronic (8–12 d) exposures have similar effects on juvenile and reproductively mature daphnids. In addition, we determined whether daphnids recover from pyriproxyfen exposures, and if recovery was dependent on age and length of exposure. Overall, the purpose of chapter two is to compare the reproductive effects of pyriproxyfen on *D. magna* and determine if *D. magna* can recover from the exposure to this juvenile hormone analog, and to better define the potential environmental impacts of acute and chronic exposures of pyriproxyfen to *D. magna* by investigating different developmental stages.

2.3 Materials and methods

2.3.1 *Daphnia magna* culture

A strain of *D. magna* has been maintained within the Environmental Toxicology program at Clemson University for about 20 years, and cultured as described previously (Baldwin et al., 2001). *Daphnia* were cultured in standard moderately hard water with a pH of 8.2–8.4 and a 16:8 light: dark cycle at 20–22^{0}C. Adult *D.magna* were fed 6 x10^6 *Pseudokirchneriella subcapitata* per daphnid/d supplemented with 0.25 mg dry weight of

blended TETRAFIN fish flakes (catalog # 46798-16140; Tetra Holding Inc., VA) in a 50 mL aqueous suspension.

2.3.2 Male production assays: pyriproxyfen concentration–response

Fourteen d-old female *D. magna* (n = 10) were placed in individual 50 mL glass beakers containing 40 mL of moderately hard water. Daphnids were exposed to pyriproxyfen (99% purity; FLUKA, analytical, Chemie, Buchs, Switzerland) dissolved in absolute ethanol provided to *D. magna* cultures at 0.02% of the media. The untreated group received only absolute ethanol at 0.02%. Culture media was changed every other day.

The sex of each neonate following pyriproxyfen exposure at 0 – 1245 pM (0–400 ng L^{-1}) was assessed after each brood based on the length of the first antennae (Olmstead and LeBlanc, 2003) using a dissecting microscope (American Optical-150W haloid cold light source). Male production and overall fecundity was assessed for the first four broods, which takes approximately 12 d. However, the first brood was eliminated from the data because the presence of males in this brood is sporadic and the first brood is often exposed to pyriproxyfen after the specific developmental timeframe necessary to alter the sex of the developing egg (Olmstead and Le- Blanc, 2002; Kato et al., 2011).

2.3.3 Male production assays:

Effects of age on male production adult female daphnids (n = 10) of different ages (7, 14, 21-d old) were exposed to pyriproxyfen at 155 pM (50 ng L^{-1}). This is a low but

effective concentration of pyriproxyfen, near the EC50 for inducing male production, and within the estimated environmental concentrations of 90–290 pM at aquatic depths of 1–6 feet (Serafini, 2001), and lower than measured concentrations (up to 1245 pM) 24 h after treatment of a rice field plot (Schaefer and Miura, 1990). Seven-d old adult females just prior to producing their initial brood, 14-d old adult females near their reproductive peak, and 21-d old adult females that are typically showing reduced fecundity because of their advanced age were individually exposed to pyriproxyfen. The sex of each neonate following pyriproxyfen exposure was assessed based on the length of the first antennae (Olmstead and LeBlanc, 2003) in broods 2–4 as described above. The ratio of males to females were examined instead of absolute numbers as the 7-d old and 21-d old daphnids typically produce less offspring than the 14-d old female daphnids, which makes direct comparisons problematic.

2.3.4 Acute and chronic pyriproxyfen exposure:

Acute and chronic effects of pyriproxyfen in *D.magna* was investigated by comparing juvenile and adult parthenogenetic female daphnids at age 3 d (juvenile) or 10 d old (reproductively mature) that were exposed to 155 pM (50 ng L^{-1}) pyriproxyfen for 0, 2, 4, 8, or 12 d. The overall fecundity and male production of the unexposed and exposed daphnids was followed for 18 d after the initial exposures in the 3 d old daphnids, and for 16 d following the initial exposures in the 10 d old daphnids. Initial reproduction (or delayed onset of reproduction), initial production of males, and the cessation of male production were also noted. The purpose of this experiment was to

assess the persistent effects of pyriproxyfen on fecundity and male production, and to determine the ability of daphnids to recover from pyriproxyfen exposures of different time lengths.

2.3.5 Statistics

Statistical differences were determined by ANOVA followed by Dunnett's test using GraphPad Prism Version 4.3 (GraphPad Software La Jolla CA, USA), except when multiple different groups were compared and then statistical differences were determined by ANOVA followed by Tukey's multiple comparison tests using Graph-Pad Prism. EC50 values were determined from log transformed data using GraphPad statistical software as described previously (Baldwin and Roling, 2009).

2.4. Results

2.4.1. Pyriproxyfen concentration–response

Pyriproxyfen is known to induce male production in cladoceran species (Oda et al., 2005b) with an EC50 of approximately 170–310 pM in *D. magna* (Olmstead and LeBlanc, 2003; Matsumoto et al., 2008). However, the concentration–response curve of pyriproxyfen is steep and effective concentrations may vary by laboratory and strain (Wang et al., 2005). Pyriproxyfen decreased overall fecundity (the production of males and females) and increased male production in a concentration-dependent manner (Figure. 1). The EC50 value for male production is 158 pM (95% CI of 134–182 pM) similar to previous studies with *D. magna* (Olmstead and LeBlanc, 2003; Matsumoto et

al., 2008). The lowest observed effect concentration (LOEC) for fecundity is 78 pM (25 ng L^{-1}) (Figure. 2.1), and the LOEC for male production is 155 pM (50 ng L^{-1}). Pyriproxyfen is estimated to reach aquatic concentrations of about 300 pM following run-off after application (Serafini, 2001; Olmstead and LeBlanc, 2003). Concentrations nearly half the relevant environmental concentrations (155 pM) consistently and significantly perturbed fecundity and increased male production, and consequently we used 155 pM in our subsequent studies on the time and age-dependent effects of pyriproxyfen.

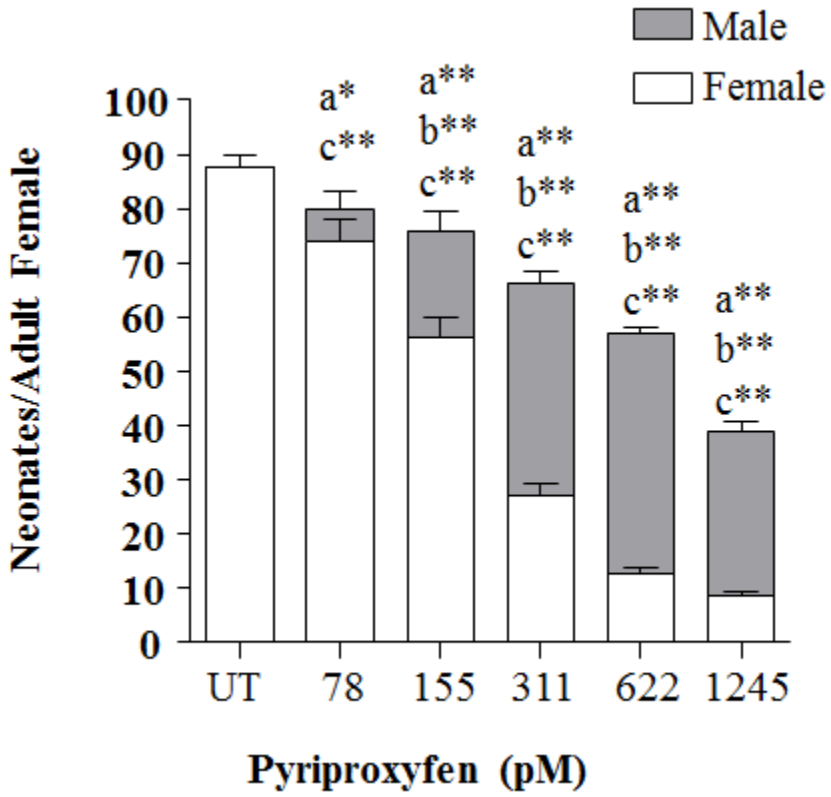


Figure. 2.1: Influence of pyriproxyfen on overall fecundity and male production. The number of male and female neonates produced per adult female. Data are shown as mean ± SEM. (a) Indicates a significant difference in the total number of neonates produced, (b) indicates a significant difference in the number of male neonates produced, and (c) indicates a significant difference in the total number of female neonates produced compared to the untreated (UT) control. Statistical differences were analyzed by ANOVA followed by Dunnett's multiple comparison test and an (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$ ($n = 10$).

2.4.2. Effects of age on male production

Exposure of 7, 14, and 21-d old female *D. magna* to 155 pM pyriproxyfen induces male production in adult daphnids regardless of age and at similar proportions (Figure. 2.2) although 14-d old daphnids typically produce the greatest number of offspring overall (data not shown). Brood-wise comparisons (examining broods 2–4 individually) indicate that older animals (21-d old) respond to pyriproxyfen quicker Appendix-A 1); producing a greater percentage and number of males in the second brood than the younger daphnids in both assays.

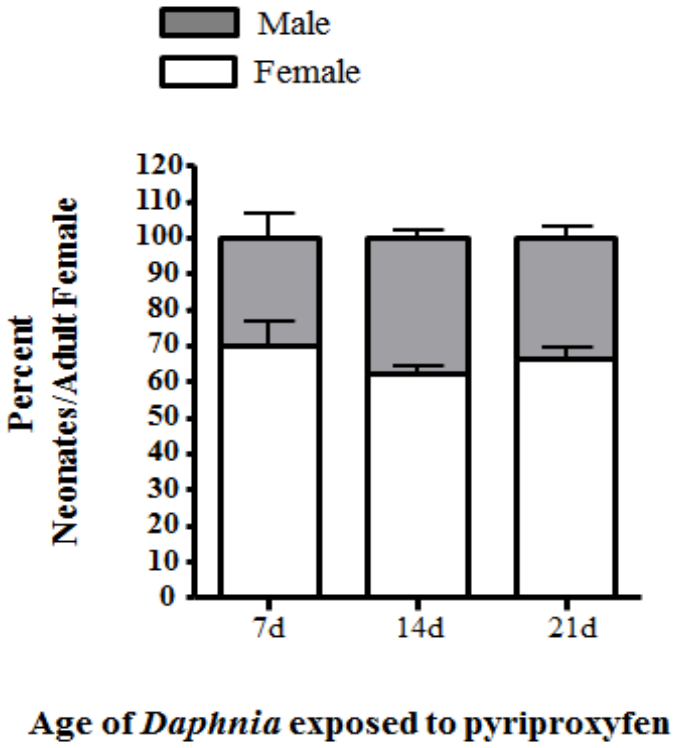


Figure. 2.2: Effects of age on the sensitivity of *D. magna* to pyriproxyfen. The percent of male and female neonates per adult female were quantified at different ages. Data are shown as mean ± SEM. Statistical differences were analyzed by ANOVA followed by Dunnett's multiple comparison test (n = 10).

2.4.3. Acute and chronic pyriproxyfen exposure

The acute and chronic effects of pyriproxyfen in juvenile and adult female parthenogenetic *D.magna* were compared. Juvenile *Daphnia* (3-d old) exposure to 155 pM pyriproxyfen for 0, 2, 4, 8, or 12-d perturbed male production, delayed the onset of reproduction, and reduced overall fecundity in a time-dependent manner. Pyriproxyfen increased the production of males after four or more days of exposure (Figure. 2.3A). The 2-d exposures produced very few males (only one adult female produced three males) and the 2-d exposure group data were significantly different from the groups exposed for more than 2 d ($p < 0.001$). Overall, the effects of pyriproxyfen on male production in juveniles are not efficacious. Pyriproxyfen-treated females produced no more than six males per female, and male production was primarily relegated to the early broods even when exposure occurred well into adulthood with the exception of the daphnids exposed for 12 d (Figure. 2.3A).

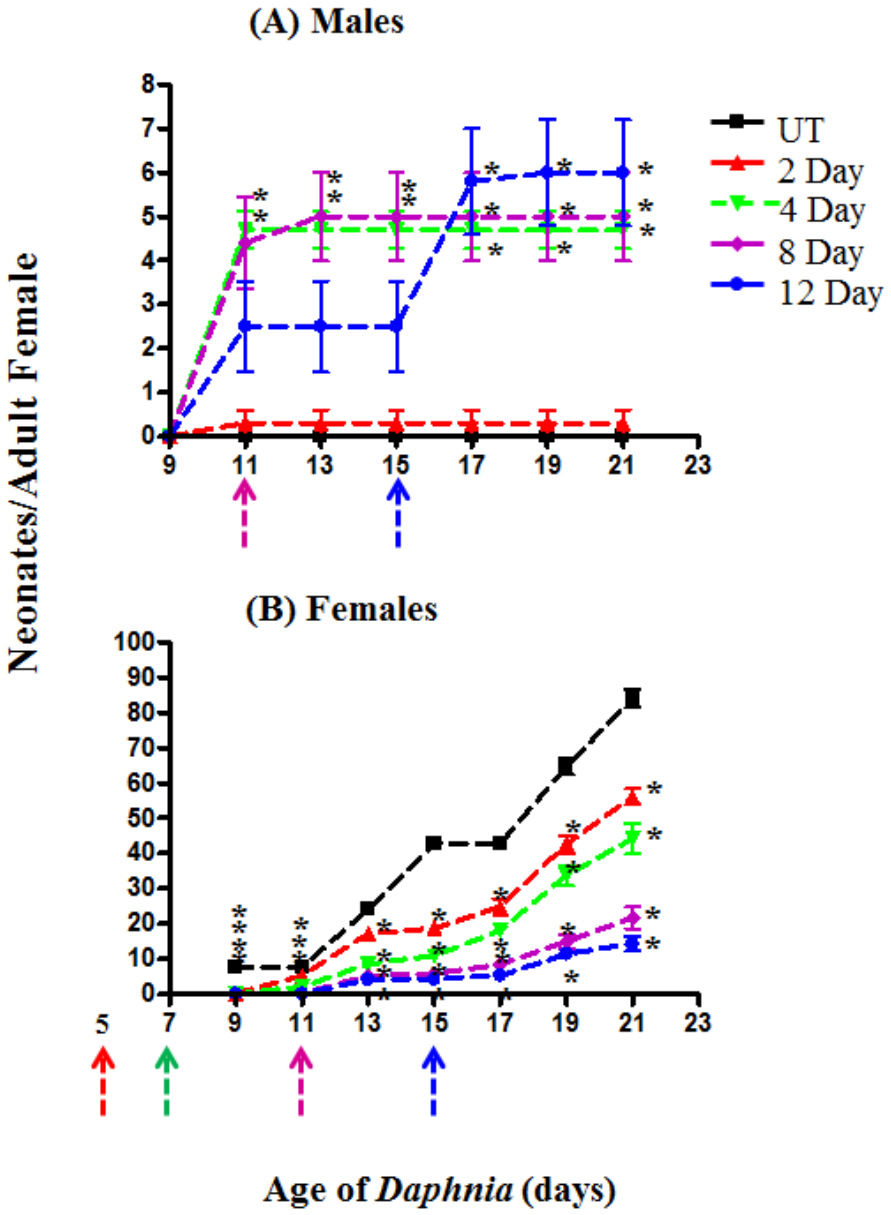


Figure. 2.3: Temporal effects of periodic exposure to pyriproxyfen on reproduction in three d old *Daphnia magna:* Female *Daphnia magna* (3-d old) were exposed to 155 pM pyriproxyfen for 2, 4, 8 or 12 d and reproduction quantified to determine the time-dependent effects of pyriproxyfen on the number of male (A) and female neonates (B) produced. Data are shown as mean $\pm$ SEM. Statistical differences were analyzed by ANOVA followed by Tukey's multiple comparison test and an (*) indicates $p < 0.001$ compared to the untreated (UT) *Daphnia* (n = 10). Colored arrows indicate the age in which pyriproxyfen exposure is withdrawn (2, 4, 8 and 12 d exposures).

The most potent effect of pyriproxyfen exposure to juveniles is on female production and overall fecundity. Pyriproxyfen reduced initial brood size and delayed reproduction (Figure. 2.3B; $p < 0.001$ ANOVA for 9-d old daphnids) relative to the unexposed daphnids, indicating that any exposure (acute or chronic) to pyriproxyfen slows maturity and reduces initial fecundity. *D. magna* usually deposit eggs in the brood chamber at approximately 6–7 d of age. The eggs develop into neonates over a 2–3 d period and are released at first molt, which occurs at about day nine (Olmstead and LeBlanc, 2002; Kato et al., 2011).

We observed that the pyriproxyfen-exposed daphnids showed a delay in the release of eggs to the brood chamber, and in turn the pyriproxyfen-exposed daphnids did not reproduce until day 11 while unexposed daphnids reproduced by day nine (Figure. 2.3). Consequently, broods 1–3 showed the most pronounced effects from pyriproxyfen exposure (Figure. 2.3; Appendix. A. 2. A).

There is also a significant exposure time-dependent decrease in the production of females and overall fecundity following pyriproxyfen exposure (Figure. 2.3B). The chronic exposures (more than 4 d) caused significantly lower fecundity compared to the acute exposures. Each increase in exposure length caused a statistically greater reduction in fecundity than the corresponding treatments except when comparing the daphnids exposed for 8 d to the daphnids exposed for 12 d. These groups show nearly equal drops in fecundity (statistics not shown; ANOVA followed by Tukey's). Typically, the longer the exposure to pyriproxyfen the greater the decline in fecundity with an 80% decrease in fecundity in daphnids exposed 12 d compared to unexposed daphnids (Figure. 2.4).

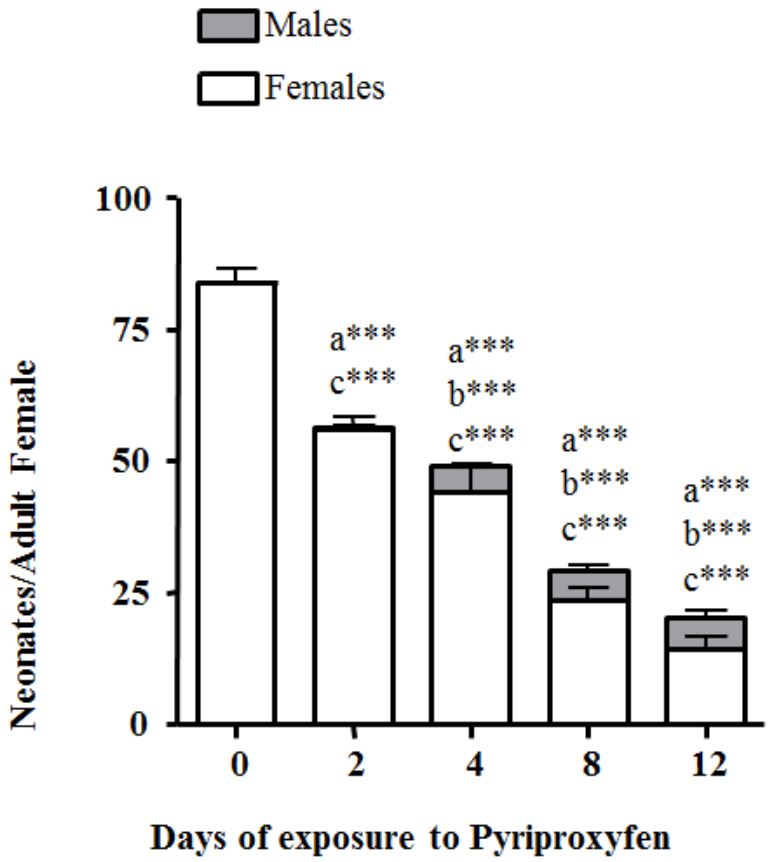


Figure. 2.4: Overall effects of temporary exposures to pyriproxyfen on reproduction in 3 d old *Daphnia magna:* Female *Daphnia magna* that were 3-d old were exposed to 155 pM pyriproxyfen for 2, 4, 8, or 12 d and reproduction monitored for 18 d. Data on the number and sex of the neonates produced by pyriproxyfen-exposed *Daphnia* are shown as mean $\pm$ SEM. (a) Indicates a significant difference in the total number of neonates produced, (b) indicates a significant difference in the number of male neonates produced, and (c) indicates a significant difference in the total number of female neonates produced compared to untreated (UT) *Daphnia magna*. Statistical differences were analyzed by ANOVA followed by Dunnett's multiple comparison test and an (*) indicates $p < 0.05$, (**) indicates $p < 0.01$ (***) indicates $p < 0.001$ ($n = 10$).

Reproduction appeared to recover in the 2- and 4-d pyriproxyfen exposure groups by day 17 (approximately 10-d after pyriproxyfen withdrawal) with reproductive slopes similar to the untreated *Daphnia* (Figure. 2.3B). Reproduction within the first three broods was clearly reduced at all exposure lengths and in a time-dependent manner. However, the daphnids exposed for only 2–4 d fully recovered during the last three broods (Appendix-A.2). Daphnids exposed for 8 or 12-d did not recover. Daphnids exposed for 8-d showed lower fecundity and daphnids exposed for 12-d showed lower fecundity coupled with continued male production (Appendix A.2.B).

Overall, the primary effect of pyriproxyfen on juvenile daphnids is lower fecundity as pyriproxyfen reduced the total number of neonates and the number of females produced by 3-d old *D. magna* in a time-dependent manner (Figure.2. 4). Some of effects of pyriproxyfen on fecundity can be explained by delayed development (Figure. 2.3). The effect of juvenile hormones on daphnid reproduction and juvenile development has not been as well documented as shown here to our knowledge.

Similarly, we determined the time-dependent influence of 0, 2, 4, 8, and 12-d exposures of 155 pM pyriproxyfen on 10-d old *D. magna*. Male production occurred six days after the initial exposure in all the treatment groups (Figure. 2.5A). Initial male production was equal in all groups; however, over the course of the assay, male production increased in a time-dependent fashion (Figure.2. 5A). The *D. magna* exposed for 2–4 d demonstrated relatively quick recovery compared to the 8 or 12 d exposure groups as 2–4 d exposed daphnids quit producing males about 2–4 d after the pyriproxyfen exposure ceased (Figure. 2.5A). This finding indicates that the male-

producing effects of pyriproxyfen are reversible, and longer exposures significantly increase the likelihood of an adverse outcome.

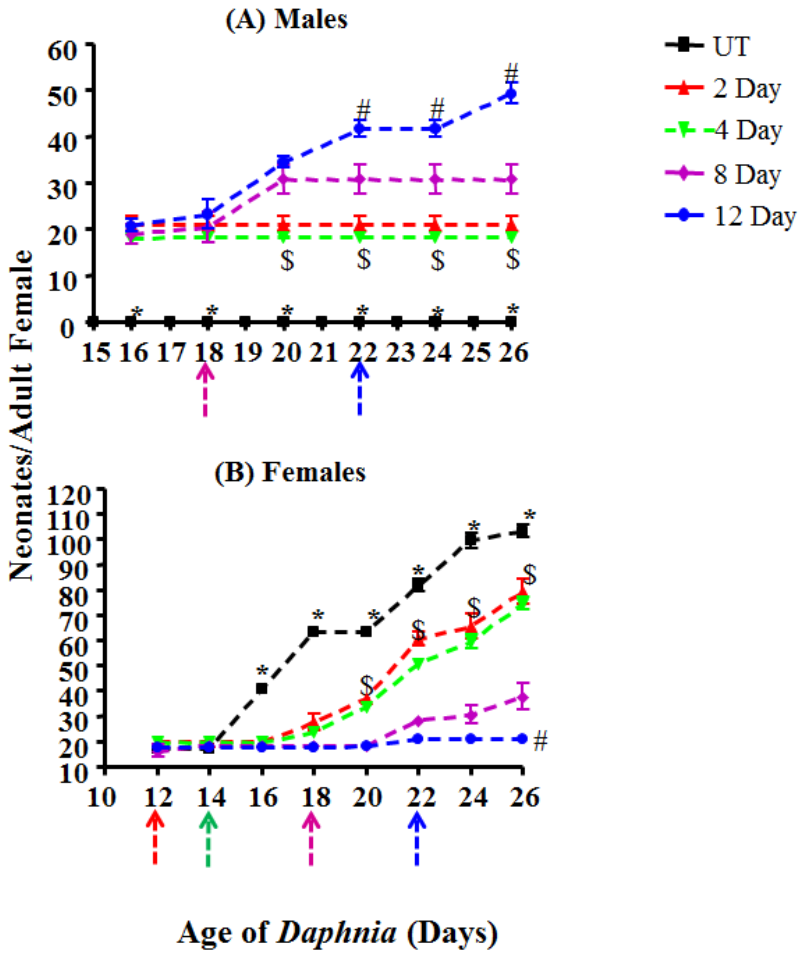


Figure. 2. 5: Temporal effects of periodic exposure to pyriproxyfen on reproduction in ten-d old *Daphnia magna:* Female *Daphnia magna* (10-d old) were exposed to 155 pM pyriproxyfen for 2, 4, 8 or 12 d and reproduction quantified to determine the time-dependent effects of pyriproxyfen on the number of male (A) and female neonates (B) produced. Data are shown as mean $\pm$ SEM. Statistical differences from the untreated daphnids were determined by ANOVA followed by Tukey's multiple comparison test. An (*) indicates all treated groups are different from the untreated (UT) group ($p < 0.001$), ($) indicates a significant difference between the groups treated for 2-4 d and the other groups, and (#) indicates a significant difference between the group treated for 12-d and all the other groups ($p < 0.001$) ($n = 10$). Colored arrows indicate the age in which pyriproxyfen exposure is withdrawn.

While male production increased, female production decreased (Figure. 2.5B) providing fewer *Daphnia* for future parthenogenic reproduction. Female production decreased in a time-dependent manner (Figure. 2.5B); however, total fecundity was only perturbed following the chronic (8–12 d) exposures (Figs. 2. 5 and 2. 6). The data suggest that the primary effect on female production was early but not immediate, about 6–8 d after exposure. Recovery was observed in the 2–4 d exposure groups shortly thereafter (Figure. 2.5B).

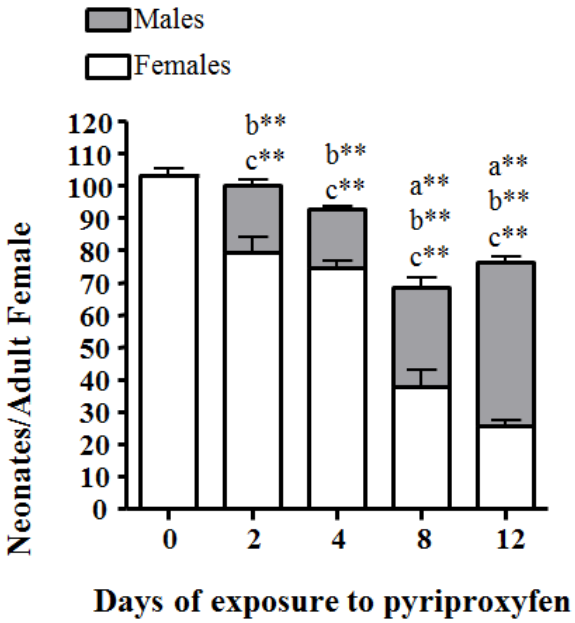


Figure. 2.6: Overall effects of temporary exposures to pyriproxyfen on reproduction in ten-d old *Daphnia magna:* Female *Daphnia magna* that were ten-d old were exposed to 155 pM pyriproxyfen for 2, 4, 8, or 12 d and reproduction monitored for 16 d. Data on the number and sex of the neonates produced are shown as mean $\pm$ SEM. (a) Indicates a significant difference in the total number of neonates produced, (b) indicates a significant difference in the number of male neonates produced, and (c) indicates a significant difference in the total number of female neonates produced compared to untreated (UT) *Daphnia magna*. Statistical difference were analyzed by ANOVA followed by Dunnett's multiple comparison test and an (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$ ($n = 10$).

Therefore, we separated the data and examined male production, female production, and overall fecundity during the first three and last three broods. Male production does not differ between treatment groups (untreated group excluded) during the initial broods, but only the daphnids exposed for 12 d showed male production in the last three broods (Appendix. A. 3) *Daphnia* exposed to pyriproxyfen for 8 d recovered enough to quit producing males, but exposures that were up to 12 d significantly perturbed fecundity and this effect was particularly evident in the last three broods (Figure. 2. 5B; Appendix. A. 3).This finding indicates that adult daphnids can recover from acute exposures to pyriproxyfen, whereas continued exposure causes irreversible loss of fecundity.

Overall, the primary effects of pyriproxyfen on mature 10 d old daphnids are increased male production and lower fecundity (Figures. 2. 5 and 2. 6).The magnitude of the effects of pyriproxyfen on adults is time-dependent, and the total number of offspring is only perturbed in reproductively active female daphnids if exposures were longer than 4 d (Figure. 2.6). However, the sex ratios of the offspring are perturbed. The percent of male offspring exposed to pyriproxyfen for 2, 4, 8, or 12 d was 20%, 20%, 45%, and 50%, respectively (Figure. 2. 6). Adult female daphnids fully recovered less than 4 d after pyriproxyfen exposure ceased with the exception of the daphnids exposed for 12 d. In contrast, juvenile daphnids exposed for only 2–4 d needed approximately 10 d for recovery. In summary, male production is much greater in the exposed adults, but overall fecundity is perturbed more in the exposed juveniles probably due in part to delayed

maturity, indicating that pyriproxyfen has different effects on juvenile daphnids than mature daphnids.

2.5 Discussion

Acute exposures to pyriproxyfen reduced fecundity, increased time to maturity, and increased male production. This could have profound effects on the population of *Daphnia* in an aquatic ecosystem. The effect of pyriproxyfen or juvenile hormone analogs on population recovery has not been investigated; however, the effects of other pesticides such as cypermethrin, fenvalerate, and paraoxon-methyl on *Daphnia* population recovery have been investigated. Some studies have indicated that reduced brood size and increased time to reproductive maturity may not perturb intrinsic growth rate of the population (Kim et al., 2008). However, most studies indicate that perturbations in reproduction may take 1–3 generations for recovery (Liess et al., 2006; Pieters and Liess, 2006) and even longer (five or more generations) if there are other stressors such as a population in stasis, poor nutrition, competition, or delayed life history events (Liess et al., 2006; Foit et al., 2012). Competition for resources by non-reproducing males and delayed reproduction are both concerns with pyriproxyfen. These variables can also continue to perturb population structure after population numbers have recovered (Liess et al., 2006).

Interestingly, the adverse effects of pyriproxyfen exposure to juvenile and reproductively mature daphnids were different. Juvenile daphnids primarily show reduced fecundity and delayed maturity; adult daphnids show limited reduced fecundity

and much greater male production. Two-d exposures to juvenile daphnids did not induce male production with the exception of one individual and this daphnid only produced three males. We hypothesize that pyriproxyfen may not induce male production in juvenile daphnids because the ovaries are not mature enough to produce offspring. The receptors or requisite transcription factors necessary to produce males may not be expressed until near reproductive maturity. Interestingly, the male producing effects of juvenoids including pyriproxyfen on reproductively mature *Daphnia* are probably unique to Cladocera (Olmstead and LeBlanc, 2003; Oda et al., 2005a). However, the effects of pyriproxyfen on juveniles are similar to those observed in insects and ticks in which reproductive maturity or metamorphosis is inhibited, and the individuals are kept in the juvenile stages (Ishaaya and Horowitz, 1992; Fathpour et al., 2007).

The effects of 155 pM pyriproxyfen on adults were significant, but reproduction was perturbed more in the pyriproxyfen exposed juvenile *Daphnia* compared to the adult *Daphnia*, especially following the shorter exposures. Exposed juveniles took longer to recover and their initial reproduction was significantly delayed (Figure. 3). Mature daphnids showed no loss of fecundity in the first three broods, just a significant increase in male production (Appendix. A.3). Furthermore, only the mature daphnids exposed for a longer period of time demonstrated reduced fecundity (Figure. 2. 6). However, that does not take into account multigenerational effects male production has on population numbers as only the females can reproduce parthenogenically and accordingly produce high numbers of offspring. Therefore, *Daphnia* numbers are likely to drop significantly

even in the adult, acute exposure groups because of reduced reproductive capacity of future generations.

Other crustaceans (and sensitive beneficial insects) may also be adversely affected as pyriproxyfen has been shown to decrease survival rates in decapod species such as shrimp (Tuberty and McKenney, 2005), and induce abnormal ovarian function in Island Red Crab (Linton et al., 2009). Pyriproxyfen applied to rice plots at 0.05 and 0.11 kg active ingredient/hectare was persistent in the water column at 0.5 m depths for 2 d. Residues were detected at 400 ng L^{-1} (1245 pM) after 24 h and in turn caused a decline in the populations of Podocopa (subclass of ostracods), Odonata (damselflies and dragonflies), and Cladocera (Schaefer and Miura, 1990). Furthermore, Odonata and Chironomidae died during pupal-adult ecdysis in subsequent tests (Schaefer and Miura, 1990). Pyriproxyfen also produced minor morphogenic aberrations in Odonata and reproductive suppression in Cladocera and Ostracoda (Schaefer et al., 1988).

Given that pyriproxyfen has an average half-life of 5.04 d in water (Sullivan, 2000), and 16–21 d in sediments (WHO, 2008), the effects on Cladocera or other non-target crustaceans may be mitigated due to recovery. Multiple seasonal sprayings can be helpful in controlling pests with multiple generations per season and are sometimes suggested. However, follow-up spraying may have severe effects on aquatic communities because reproduction is perturbed in a time-dependent manner, and juvenile daphnids, which already show delayed development following acute exposures, may not recover from chronic exposures (Figures. 2. 4 and 2. 6). Recent data indicate that follow-up

spraying is not necessary as pyriproxyfen is effective for five months and even longer if used in combination with insecticide spinosad (Chen et al., 2008; Darriet et al., 2010).

We also investigated the age-dependent effects of pyriproxyfen on male production at 155 pM, examining adolescent (7-d old), reproductively mature (14-d old), and reproductively declining daphnids (21-d old). These assays reveal that all adolescent and adult stages are sensitive to the male producing effects of pyriproxyfen. Overall, the data indicate that age has little effect on the ratio of male/female production. An interesting and repeatable effect observed is increased production of males in brood 2 in the oldest (21-d old) daphnids compared to the younger daphnids (Appendix-A.1). Previous research indicates that *D. pulex* offspring become more male-biased with maternal age during overcrowding (Fitzsimmons and Innes, 2006). We did not observe an increase in the male production or the male/female ratio over several broods, but did observe an increased initial sensitivity to a stressor that induces male production. Increased male production in older daphnids may provide a mechanism by which a population of *Daphnia* can respond to a key stressor by producing males, while some younger individuals may continue to produce primarily parthenogenic clones to increase or stabilize population numbers.

In conclusion, the male producing effects of juvenoids including pyriproxyfen on *Daphnia* are probably unique to Cladocera (Olmstead and LeBlanc, 2003; Oda et al., 2005b). However, the effects of pyriproxyfen on juveniles are similar to those observed in insects in which reproductive maturity is inhibited (Ishaaya and Horowitz, 1992; Fathpour et al., 2007). Furthermore, pyriproxyfen suppressed fecundity more and

recovery took longer in the juvenile daphnids compared to the mature daphnids. Daphnids, especially adults, can recover from the reproductive toxicity elicited by pyriproxyfen after short-term exposures. However, the effects of pyriproxyfen on non-target *Daphnia* species (Schaefer et al., 1988) are substantial and may include other arthropods at environmentally relevant concentrations (Schaefer and Miura, 1990). Consequently, our data indicate that while pyriproxyfen is a relatively safe pesticide from which individuals can recover, its effects can occur at concentrations within the expected environmental concentrations. Further, the effects are time-dependent and individuals may not recover from chronic exposures.

2.6 References

Baldwin, W.S., Roling, J.A., 2009. A concentration addition model for the activation of the constitutive androstane receptor by xenobiotic mixtures. Toxicol. Sci.107, 93–105.

Baldwin, W.S., Bailey, R., Long, K.E., Klaine, S., 2001. Incomplete ecdysis is an indicator of ecdysteroid exposure in *Daphnia magna*. Environ. Toxicol. Chem. 20, 1564–1569.

Carpenter, S., Kitchell, J., Hodgson, J., Cochran, P., Elser, J., Elser, M., Lodge, D., Kretchmer, D., He, X., Von, E., 1987. Regulation of lake primary productivity by food web structure. Ecology 68, 1863–1876.

Chen, C., Andy-Tana, W., Loke, S., Lee, H., Yasmin, A., Sofian-Azirun, M., 2008. Effectiveness of pyriproxyfen-controlled release block against larvae of *Aedes* (*Stegomyia*) *aegypti* in Kuala Lumpur, Malaysia. Dengue Bull. 32, 199–206.

Chen, W., Fu, X., Dong, B., Wang, Y., Shiah, S., Moore, D., Huang, W., 2012. Neonatal activation of the nuclear receptor CAR results in epigenetic memory and permanent change of drug metabolism in mouse liver. Hepatology 56, 1499–1509.

Dang, Z., Cheng, Y., Chen, H.M., Cui, Y., Yin, H.H., Traas, T., Montforts, M., Vermeire, T., 2012. Evaluation of the *Daphnia magna* reproduction test for detecting endocrine disruptors. Chemosphere 88, 514–523.

Darriet, F., Marcombe, S., Etienne, M., Yébakima, A., Agnew, P., Yp-Tcha, M., Corbel, V., 2010. Field evaluation of pyriproxyfen and spinosad mixture for the control of insecticide resistant *Aedes aegypti* in Martinique (French West Indies). Parasites & Vectors. 3:88.

Deng, H., Lynch, M., 1996. Change of genetic architecture in response to sex. Genetics 143, 203–212.

Ebert, D., 2005. Ecology, epidemiology, and evolution of parasitism in *Daphnia*. National Center for Biotechnology Information, Bethesda, MD.

Fathpour, H., Noori, A., Zeinali, B., 2007. Effects of a juvenoid pyriproxyfen on reproductive organ development and reproduction in German Cockroach (*Dictyoptera: Blattellidae*). Iranian J. Sci. Technol. Trans-A 31, 89–98.

Fitzsimmons, J.M., Innes, D.J., 2006. Inter-genotype variation in reproductive response to crowding among *Daphnia pulex*. Hydrobiologia 568, 187–205.

Foit, K., Kaske, O., Leiss, M., 2012. Competition increases toxicant sensitivity and delays the recovery of two interacting populations. Aquat. Toxicol. 106–107, 25–31.

Gaedke, U., Straile, D., 1998. Daphnids: Keystone species for the pelagic food web structure and energy flow.A body size-related analysis linking seasonal changes at the population and ecosystem levels. Adv. Limnol. 53, 587–610.

Ishaaya, I., Horowitz, A., 1992. Novel phenoxy juvenile hormone analog (pyriproxyfen) suppresses embryogenesis and adult emergence of sweetpotato whitefly (*Homoptera: Aleyrodidae*). J. Econ. Entomol. 85, 2113–2117.

Katagi, T.,Takahashi, N., 1994.Hydrolysis of S-31183 in Buffered Aqueous Solutions. Sumitomo Chemical Company.

Kato, Y.,Kobayashi, K., Watanabe, H., Iguchi, T., 2011. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a doublesex gene in the sex-determining pathway. PLoS Gen.7, e1001345.

Kim, Y., Jung, J., Oh, S., Choi, K., 2008. Aquatic toxicity of cartap and cypermethrin to different life stages of *Daphnia magna* and *Oryzias latipes*. J. Environ. Sci. Health Part-B, 56–64.

Kleiven, O.T., Larsson, P., Hobæk, A., 1992. Sexual reproduction in *Daphnia magna* requires three stimuli. OIKOS 65, 197–206.

Koch, U., Von Elert, E., Straile, D., 2009. Food quality triggers the reproductive mode in the cyclical parthenogen *Daphnia* (Cladocera). Oecologia 159, 317–324.

Liess, M., Pieters, B., Duquesne, S., 2006. Long-term signal of population disturbance after pulse exposure to an insecticide: Rapid recovery of abundance, persistent alteration of structure. Environ. Toxicol. Chem. 25, 326–1331.

Linton, S., Lauren, D., Harman, L., 2009. Potential endocrine disruption of ovary synthesis in the Christmas Island red crab *Gecarcoidea natalis* by the insecticide pyriproxyfen. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 154, 289–297.

Matsumoto, T.,Ikuno, E., Itoi, S., Sugita, H., 2008. Chemical sensitivity of the male daphnid, *Daphnia magna*, induced by exposure to juvenile hormone and its analogs. Chemosphere 72, 451–456.

Maynard, L., Houffschmitt, P., Lebreux, B., 2001.Field efficacy of a 10% pyriproxyfen spot-on for the prevention of flea infestations on cats. J. Small Anim. Pract. 42, 491–494.

Meola, R., Meier, K., Dean, S., Bhaskaran, G., 2000. Effect of pyriproxyfen in the blood diet of cat fleas on adult survival, egg viability, and larval development. J. Med. Entomol. 37, 503–506.

Meylan, W.M., Howard, P.H., Boethling, R.S., Aronson, D., Printup, H., Gouchie, S., 1999. Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. Environ. Toxicol. Chem. 18, 664–672.

Michael, K.R., 2005.Advances in the control of *Ctenocephalides felis* (cat flea) on catsand dogs. Trends Parasitol. 21, 232–236.

Nichols, J., Breen, M., Denver, R., Distefano, J., Edwards, J., Hoke, R., Volz, D., Zhang, X., 2011.Predicting chemical impacts on vertebrate endocrine systems. Environ. Toxicol. Chem. 30, 39–51.

Oda, S.,Tatarazako, N., Watanabe, H., Morita, M., Iguchi, T., 2005a. Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. Chemosphere 61, 1168–1174.

Oda, S.,Tatarazako, N., Watanabe, H., Morita, M., Iguchi, T., 2005b. Production of male neonates in four cladoceran species exposed to a juvenile hormone analog, fenoxycarb. Chemosphere 60, 74–78.

Organization for Economic Co-operation and Development., 2012, *Daphnia magna* reproduction test, OECD Guidelines for the Testing of Chemicals. OECD Publishing.

Olmstead, A.W., LeBlanc, G.A., 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. J. Exp. Zool. 293, 736–739.

Olmstead, A.W., LeBlanc, G.A., 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environ. Health Perspect. 111, 919–924.

Pieters, B., Liess, M., 2006. Population developmental stage determines the recovery potential of *Daphnia magna* populations after fenvalerate application. Environ. Sci.Technol. 40, 6157–6162.

Schaefer, C., Miura, T., 1990. Chemical persistence and effects of S-31183, 2-1- methyl-2-(4-phenoxyphenoxy) ethoxy pyridine, on aquatic organisms in field tests. J. Econ. Entomol. 83, 1768–1776.

Schaefer, C., Miura, T., Dupras, E., Mulligan, F., Wilder, W., 1988. Efficacy, non-target effects and persistence of S-31183, a promising mosquito (*Diptera-Culicidae*) control agent. J. Econ. Entomol. 86, 1648–1655.

Serafini, M., 2001., Pyriproxyfen – Registration of a Major Change of Label for Esteem 3/01. In: Division of Solid & Hazardous Materials, Bureau of Pesticides Management P.P.R.S. New York State, Department of Environmental Conservation, New York.

Smith, A., Acharya, K., Jack, J., 2009. Overcrowding, food and phosphorus limitation effects on ephippia production and population dynamics in the invasive species *Daphnia lumholtzi*. Hydrobiologia 618, 47–56.

Steginsky, C., Powell, J., Chang, J., 1994. Characterization of 14C Residues in Bluegill Sunfish Treated With 14C Pyriproxyfen Data Package Report No. 156629-N, DPR No. 52080-0041 (Unpublished). Sumitomo Chemical Company.

Sullivan, J., 2000. Environmental fate of pyriproxyfen. California Department of Pesticide Regulation.

Sullivan, J., Goh, K., 2008. Environmental fate and properties of pyriproxyfen. J. Pest. Sci. 33, 339–350.

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., Iguchi, T., 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53, 827–833.

Tuberty, S.R., McKenney, C.L., 2005. Ecdysteroid responses of estuarine crustaceans exposed through complete larval development to juvenile hormone agonist insecticides. Integr. Comp. Biol. 45, 106–117.

USEPA, (United States Environmental Protection Agency), 2007 (15 April 2009). Draft List of Chemicals for Initial Tier 1 Screening. EPA-HQ-OPPT-2004 -0109; FRL-8399-7. Draft list of initial pesticide active ingredients and pesticide inerts to be considered for screening under the federal food, drug, and cosmetic act USEPA, Federal Register74: 71. http://www.epa.gov/endo/pubs/final_list_frn_041509.pdf

Wang, H.Y., Olmstead, A.W., Li, H., LeBlanc, G.A., 2005. The screening of chemicals for juvenoid-related endocrine activity using the water flea *Daphnia magna*. Aquat. Toxicol. 74, 193–204.

WHO, (World Health Organization). 2008. Chemical hazards in drinking-water – pyriproxyfen. Pyriproxyfen in drinking-water: use for vector control in drinking-water sources and containers. WHO/HSE/AMR/0803/9, Geneva, 1–5. http://www.who.int/water_sanitation_health/dwq/chemicals/pyriproxyfen_2add_feb%202008. pdf

CHAPTER THREE

ARACHIDONIC ACID ENHANCES REPRODUCTION IN *DAPHNIA MAGNA* AND MITIGATES CHANGES IN SEX RATIOS INDUCED BY PYRIPROXYFEN

3.1 ABSTRACT

Arachidonic acid (AA) is one of only two unsaturated fatty acids preferentially retained in the ovaries of crustaceans such as *Daphnia*. We hypothesized that AA is associated with reproduction and environmental sex determination in *Daphnia* because it is retained in the ovaries, and it represses HR97 activity, a nuclear receptor preferentially expressed in the ovaries that is weakly activated by the male producing pesticide, pyriproxyfen. Reproduction assays with different fatty acids indicate that only AA alters female/male sex ratios in the presence of pyriproxyfen. However, the primary effect was not decreased male production, but increased female production, and this reproductive effect only occurred during a restricted algal diet. Next, we tested whether enriching an algal diet (*P.subcapitata* has moderate AA levels and *C. vulgaris* has poor AA levels) with AA enhances overall reproduction and sex ratios. AA enrichment of a *C. vulgaris* diet enhances fecundity at 1.0 and 4.0 μM by 30% in the presence and to 40% absence of pyriproxyfen. This indicates that AA is crucial in reproduction regardless of environmental sex determination. Furthermore, *P. subcapitata* is provides a threshold concentration of AA needed for reproduction. Diet switch experiments from *P. subcapitata* to *C. vulgaris* mitigate some but not all the effects of AA, when compared to a *C. vulgaris* only diet, suggesting that some AA provided by *P. subcapitata* is retained.

In summary, a threshold concentration must be met to provide for maximal reproduction in *Daphnia magna* as AA supplementation increases reproduction and represses pyriproxyfen-induced environmental sex determination in two different restricted diets. Further, a diet rich in AA may provide protection from some reproductive toxicants such as the juvenile hormone agonist, pyriproxyfen.

3.2 Introduction:

The cladoceran, *Daphnia magna,* is an aquatic indicator species typically found in freshwater ponds (Carpenter et al., 1987), where they are mid-level consumers and a food source for small fish (Gaedke and Straile, 1998). *Daphnia* have a short maturation time and typically produce large broods of females through parthenogenesis, building rich populations in a short time span (Anderson and Jenkins, 1942). However, under stressful conditions such as overcrowding, scarcity of food (Smith et al., 2009), fall in ambient temperatures (Kleiven et al., 1992), and altered photoperiods (Deng and Lynch, 1996), *Daphnia* may produce males (Tatarazako et al., 2003) and switch to sexual reproduction, where a hardy ephippia (or winter egg) that can survive desiccation is produced. Methyl farnesoate, an endogenous juvenoid in *Daphnia* (Olmstead and LeBlanc, 2002) and some juvenile hormone analog pesticides, such as pyriproxyfen and fenoxycarb, can induce male production (Olmstead and LeBlanc, 2002; Oda et al., 2005; Ginjupalli and Baldwin, 2013).

The polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA; ω-6) and eicosapentaenoic acid (EPA; ω-3) are important in arthropod physiology. PUFAs,

especially highly unsaturated fatty acids, contribute to the fluidity of cell membranes in fish and cladocerans and help these organisms to withstand cold temperatures (Michael et al., 1997). Fish fed on zooplankton rich in PUFAs exhibit higher growth rates and fecundity (Verreth et al., 1994) and zooplankton grazing on PUFA rich phytoplankton exhibit higher growth rates and fecundity (Michael et al., 1997).

Though reproduction is expensive in terms of energy expenditure (Cox and Calsbeek, 2010), it is one of the principal driving forces that dictate the fitness of a species in a resource limited competitive environment. How daphnids respond and adapt to environmental cues is not clearly understood, but their efficiency in allocating limited dietary resources is crucial to survival and reproductive preferences (Meester et al., 2011). A closer look at allocation of dietary components indicates that adult daphnids preferentially accumulate the dietary PUFAs, AA and EPA, and much of these are allocated to the ovary in the late stages of oocyte maturation (Ahlgren et al., 1990; Goulden and Place, 1993; Bec et al., 2003; Wacker and Martin-Creuzburg, 2007; Taipale et al., 2011). AA is also important in the production of prostaglandins that are produced from AA through the cyclooxygenase pathway (Needleman et al., 1986). We hypothesized that dietary PUFAs are retained in the daphnid ovary because they are crucial in reproduction. AA also inhibits the novel nuclear receptor group, HR97g (Appendix.B.3), found primarily in adult ovary (Appendix.B.2) and gastrointestinal tract and weakly activated by the male-producing JHA, pyriproxyfen (Ginjupalli et al., 2011). Therefore, we also considered that a diet supplemented with PUFAs and specifically AA may help repress male production.

AA is a precursor to eicosanoids that are associated with several important physiological functions such as immune function, reproduction and ion transport (Stanley, 2006; Heckmann et al., 2008; Büyükgüzel et al., 2011). Accumulation of AA in the ovaries of marine shrimp *Penaeus semisulcatus* is associated with oocyte maturation (Ravid et al., 1999). The prostaglandin, PGE_2, a metabolite of AA from the cyclooxygenase pathway is associated with egg laying behavior in insects (Stanleysamuelson and Loher, 1986), and $PGF_{2\alpha}$ is responsible for enhanced sperm motility and ovulation in mammals (Chang et al., 1997). Therefore, accumulation of AA in the ovary is important as AA may directly influence reproduction or act through the production of eicosanoids to function as signaling molecules responsible for egg maturation or release.

Daphnia are filter feeders that preferentially consume algae as their food source. A typical daphnid diet used during environmental toxicology testing may contain *P. subcapitata*, (formerly known as *Selanastrum capricornutum*) supplemented with yeast or fish food (ASTM, 1993; Baldwin et al., 2001; Ginjupalli and Baldwin, 2013). Different algal diets contain different concentrations of saturated and unsaturated fatty acids. Fatty acid concentrations have been determined in some algal species (Brown et al., 1997; Guedes et al., 2011). For example, *Nannochloropsis oculata* contains high concentrations of AA and several ω-3 fatty acids. *Pseudokirchneriella subcapitata* contains moderate levels of these fatty acids, and *Chlorella vulgaris* contains high levels of linoleic acid, the precursor to AA, but virtually no AA (Ahlgren et al., 1990) and extremely low levels of most ω-3 fatty acids (Brown et al., 1997; Guschina and Harwood,

2006; Guedes et al., 2011). In our study, daphnids were provided algal diets that differ in fatty acid composition to help ascertain, whether high or low levels of specific unsaturated fatty acids influence reproduction and male production.

Our hypotheses are in part influenced by our discovery of a novel group of nuclear receptors (NR1L; HR97) that contain three members (HR97a, HR97b, and HR97g) (Thomson et al., 2009) of which HR97g is weakly activated by pyriproxyfen and all three receptors are inhibited by AA (Appendix. B.1) (Ginjupalli et al., 2011). The HR97 receptors are related to the HR96 group (NR1J) of receptors that are promiscuous xenobiotic receptors related to the mammalian receptors, CAR, PXR, and VDR (Karimullina et al., 2012), and the xenobiotic, triacylglycerol, and cholesterol *Drosophila* HR96 receptor (King-Jones et al., 2006; Horner et al., 2009; Sieber and Thummel, 2009; Sieber and Thummel, 2012).

The specific influence of individual fatty acids on daphnid reproduction and their regulation of fecundity and environmental sex determination are unknown. Based on the preferential accumulation of AA in daphnid ovary, its involvement in insect egg laying behavior and potentially its inhibition of HR97, we hypothesized that AA is associated with ameliorating pyriproxyfen-induced male production. The purpose of the study is to determine the influence of AA, a ω-6 unsaturated fatty acid on *Daphnia* reproduction. Therefore, adult, parthenogenic *D. magna* which are reproductively active that were exposed to fatty acids, including AA alone or in combination with pyriproxyfen to determine the influence of AA on *Daphnia* reproduction.

3.3 Materials & Methods

3.3.1 *Daphnia magna culture*:

A strain of *Daphnia magna* has been maintained in the Environmental Toxicology Program at Clemson University for about 20 years. Stock cultures of *D. magna* are maintained in an environmental chamber under a 16:8 hour light cycle at 21^{o}C in moderately hard water with a diet of 6 X 10^{6} *P. subcapitata* per adult daphnid/day supplemented with 0.25 mg dry weight of blended TETRAFIN fish flakes (catalog # 46798-16140; Tetra Holding Inc., VA) in a 50 μL aqueous suspension (Baldwin et al., 2001).

3.3.2 *Algal culture*:

Algae cultures of *P. subcapitata* were maintained in our laboratory per EPA guide lines (Miller et al., 1978). Mother cultures of *P. subcapitata* were obtained from Aquatic Bio Systems, (Fort Collins, Colorado). Mother cultures of *Nannochloropsis oculata* and *Chlorella vulgaris* were obtained from Algae Depot (Eau Claire, WI). Algae was cultured as described previously in prepared media (Miller et al., 1978) under continuous fluorescent light (Jumpstart, HydroFarms, West-Petaluma, CA, USA). All experiments were performed with *P. subcapitata* unless otherwise mentioned.

3.3.3 *Standard acute and chronic toxicity tests*:

Acute toxicity of AA was determined by exposing < 24 h neonates; (4 daphnids/beaker; n=4 beakers) to arachidonic acid in 40 mL of culture medium in 50 mL

glass beakers as described previously (Stephan, 1975). Neonates were exposed to 0.01 μM to 100 μM AA (>99% purity, CAS Number 506-32-1, Sigma-Aldrich, St. Louis, MO USA) dissolved in 100% ethanol. Control and AA treated daphnids were exposed up to (0.002%) ethanol.

The reproductive toxicity of AA was determined by exposing < 24h; neonates (n = 12) to AA in 40mL culture medium in 50mL glass beakers using standard methods (Baldwin et al., 1997). Neonates were exposed to 1.0, 2.0, or 4.0 μM arachidonic acid reconstituted in 100% ethanol for a total of 0.002% ethanol in each exposure container. Neonates were provided with the typical stock diet containing *P. subcapitata* and 0.25 mg dry weight of blended fish flakes as a 50 μL/day aqueous suspension for the 21 days of the test. The number of neonates produced during the 21 day test period was measured and reported as mean offspring/female daphnid. Neonates from the final brood were collected and a second generation chronic toxicity test was performed with the daphnids exposed transgenerationally as described previously (Baldwin et al., 1997).

3.3.4 Male Production Assays:

Male production assays (10 - 12 days long (Wang et al., 2005; Ginjupalli and Baldwin, 2013) were conducted to determine the influence of specific fatty acids (palmitic acid, 99% purity, CAS Number 506-32-1, docosahexaenoic acid, ≥98% purity, CAS Number 6217-54-5, linoleic acid, >99% purity, CAS Number 60-33-3, all purchased from Sigma-Aldrich) in combating male production induced by pyriproxyfen. Male production was induced by exposing the daphnids to 155 or 310 pM pyriproxyfen

(50 or 100 ng/L) (>99% purity; FLUKA, Buchs, Switzerland), an insecticidal juvenile hormone analog.

Ten-day old female daphnids (n = 10) were exposed to fatty acids alone or in combination with pyriproxyfen while housed as described in the chronic toxicity tests. All daphnids (controls and treated groups) were exposed up to 0.002% ethanol during the assays. To provide increased control over fatty acid exposure levels, daphnids were not provided fish food. Instead, daphnids were either fed the normal quantity of *P. subcapitata* (6×10^6 cells/adult daphnid) or half the normal quantity (3×10^6 cells/adult daphnid). Adult survival, the total number of neonates, the number of female neonates, and the number of male neonates produced from broods 2-5 (a 10 or 12 d period of time) were measured. Reproduction was determined only in neonates from broods 2-5, as fatty acid and pyriproxyfen exposure may occur after developmental sex determination in brood 1 (Olmstead and LeBlanc, 2002; Ginjupalli and Baldwin, 2013).

3.3.5 Influence of different algal diets on reproduction in adult female daphnids:

Chlorella vulgaris has extremely low levels of AA, *N. oculata* has high levels of AA, and *P. subcapitata* has moderate levels of AA (Brown et al., 1997; Guschina and Harwood, 2006; Guedes et al., 2011). Therefore, we examined the effect of each of these algal diets on survival and reproduction in 10 d old daphnids in an identical fashion as described in the male production assays. Briefly, neonates were acclimatized to their new algal diet without fish food at 6×10^6 cell/adult daphnids/day for three generations and

then fourth generation daphnids were exposed to half the regular diet (3 X 10^6 cells of algae/mL/day) at 10 d old. Survival and reproduction was quantified over the next 12 d.

3.3.6 Male production assays with Chlorella (low AA) diets:

D. magna were either acclimated to a *Chlorella* diet for four generations or were switched to a *Chlorella* only diet immediately prior to pyriproxyfen treatment. 10 d old daphnids were fed and exposed to: 1) *Chlorella* or *Chlorella* + AA, 2) *Chlorella* in combination with pyriproxyfen and AA, 3) *Chlorella* or *Chlorella* + AA immediately after switching the diet from a *P. subcapitata* based diet or 4) *Chlorella* in combination with pyriproxyfen and AA after switching from a *P. subcapitata* based diet. Survival and reproduction were monitored for the next 12 d from 4 broods (broods 2-5).

3.3.7 Neonate sex determination:

The sex of each neonate following pyriproxyfen exposure was assessed based on the length of the first antennae (Olmstead and LeBlanc, 2003; Ginjupalli and Baldwin, 2013) using a dissecting microscope (American Optical-150W haloid cold light source). Male production and overall fecundity was assessed for the first five broods, which takes approximately 12 d. However, the first brood was eliminated from the data because the presence of males in this brood is sporadic and the first brood is often exposed to pyriproxyfen after the specific developmental time frame necessary to alter the sex of the developing egg (Olmstead and LeBlanc, 2002; Kato et al., 2011a; Ginjupalli and Baldwin, 2013).

3.3.8 Statistics:

Statistical differences were determined by ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism Version 4.3 (GraphPad Software La Jolla CA, USA). Differences in survival were determined using Fisher's exact test (http://www.vassarstats.net/).

3.4 Results:

3.4.1 Acute and chronic toxicity of arachidonic acid in D. magna:

To determine future exposure concentrations, the acute and chronic toxicity of AA was evaluated using standard methods. Neonatal *D. magna* exposed to AA show no acute toxicity until treated with concentrations of at least 10 μM (Appendix.C.1).The reproductive toxicity of AA was determined using standard chronic toxicity assays and AA did not perturb reproduction at all concentrations examined (up to 4 μM; Appendix.C.2). Therefore, further studies were performed with 1 or 4 μM AA.

3.4.2 Fatty acid mediated increases in female production in the presence of pyriproxyfen:

Male production is associated with a number of factors and we hypothesized that a healthy diet or more specifically a diet full of healthy unsaturated fatty acids may repress male production, in part because AA and other UFAs are retained in the ovaries (Goulden and Place, 1993; Ravid et al., 1999). We were especially interested in the ability of AA in repressing male production, because HR97g is weakly activated by

pyriproxyfen and all three HR97 receptors are inhibited by AA (Appendix.B.1) (Ginjupalli et al., 2011). Therefore, we exposed daphnids to the JHA, pyriproxyfen (155 pM; 50 ng/L) in conjunction with several fatty acids under normal feeding conditions and dietary restriction (half the normal food). The number of males and females produced over the next 10 d was quantified. Under a typical *P. subcapitata* laboratory diet (6×10^6 cells/adult daphnid/day), none of the fatty acids at 4 μM concentrations altered pyriproxyfen-induced male production (Figure. 3.1A). When the diet was reduced to half the regular quantity *of P. subcapitata,* 4.0 μM AA increased the number of females produced. None of the other fatty acids significantly effected overall reproduction, male, or female production during pyriproxyfen exposure (Figure.3.1B).

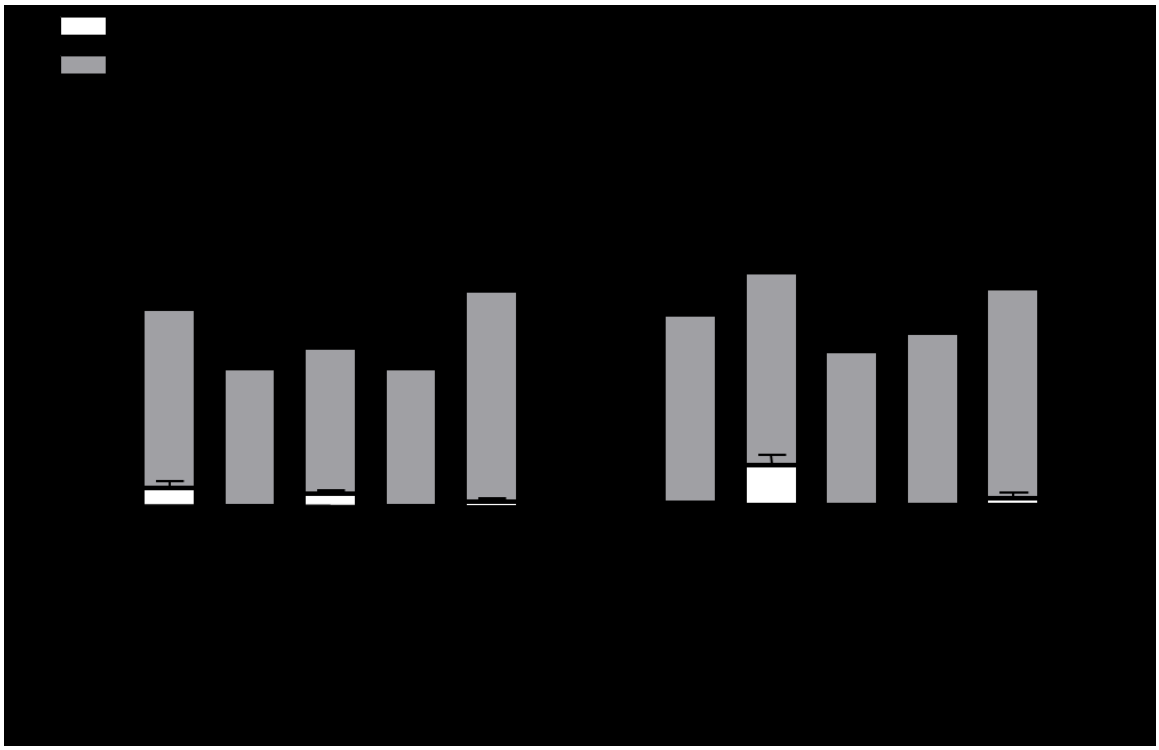


Figure. 3.1: Arachidonic acid alters pyriproxyfen-mediated male/female offspring ratio. Reproductively mature *Daphnia magna* were exposed to 155 pM pyriproxyfen or pyriproxyfen and several different dietary fatty acids at 4.0μM; arachidonic acid (AA), docosahexaenoic acid (DHA), palmitic acid (PA) and linoleic acid (LA). Production of male and female offspring was quantified from broods 2-5 during the next 10 days. (A) *D. magna* fed at normal levels of *P. subcapitata* (6 x 10^6cells/adult). (B) *D. magna* fed at half the normal level of *P. subcapitata* (3 x 10^6cells/adult). A (c) indicates a significant difference in the total number of female neonates produced as determined by one-way ANOVA followed by Dunnett's multiple comparison test (** = $p < 0.01$). Data are expressed as mean ± SEM.

3.4.3 AA alters pyriproxyfen-mediated neonatal sex ratio:

Because AA was the only fatty acid that increased female production, the role of AA as a potential inhibitor of pyriproxyfen-mediated male production was verified. A male production assay was performed exposing reproductively mature *D. magna* to two different environmentally relevant concentrations of pyriproxyfen (155 or 310 pM) (Ginjupalli and Baldwin, 2013) and AA (1.0 or 4.0 μM) either alone or in combination while being fed half the typical number of *P. subcapitata* cells/day. The *Daphnia* exposed to AA alone did not show any significant changes in the total number of neonates produced when compared to untreated *Daphnia* (Figure. 3.2A). *Daphnia* that were only exposed to pyriproxyfen exhibited a dose-dependent decrease in overall fecundity and a corresponding increase in ratio of male neonates produced (Figure. 3.2B). AA exposure significantly affected fecundity and sex ratios in favor of female neonates in daphnids co-exposed with pyriproxyfen (Figure. 3.2C/D) confirming the previous experiment.

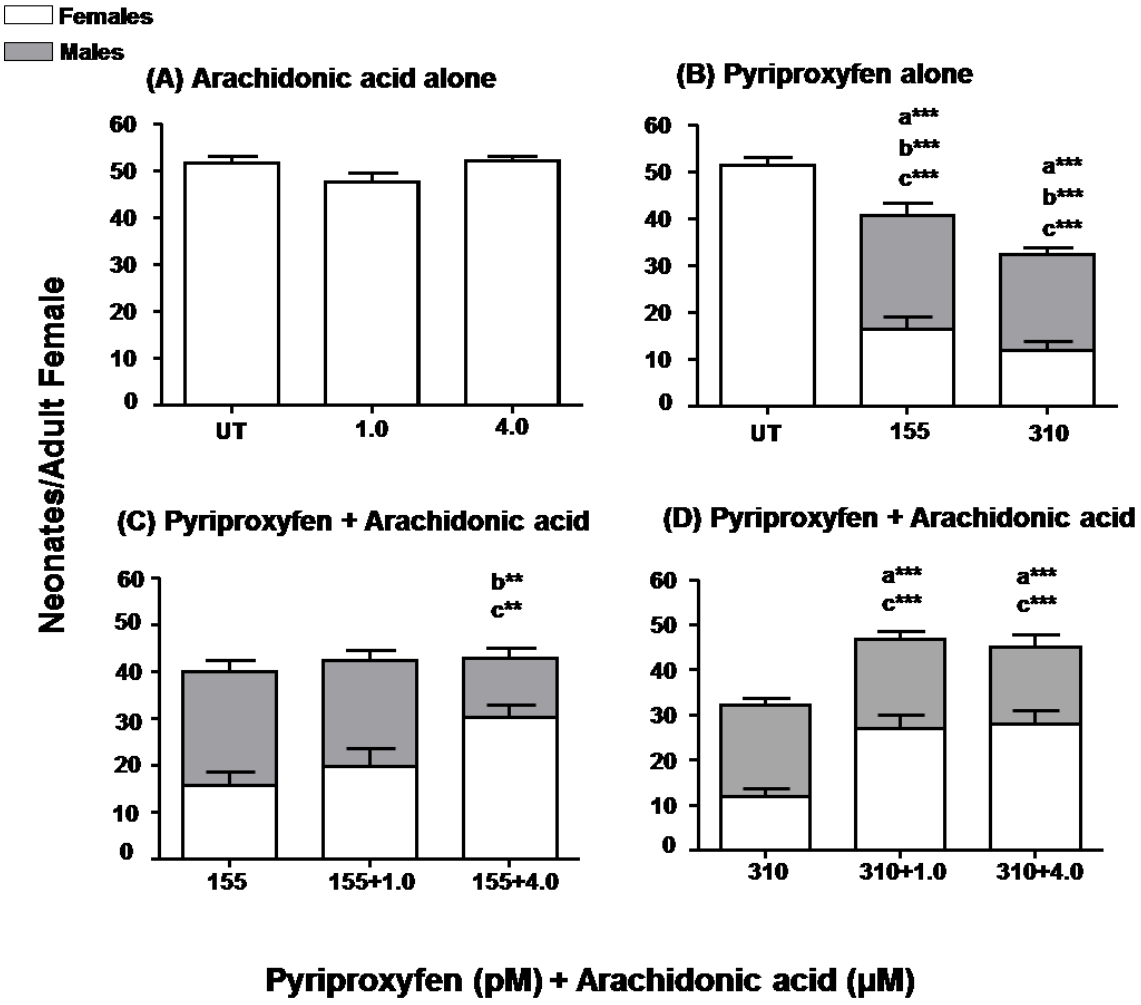


Figure. 3.2: Arachidonic acid alters pyriproxyfen-mediated male/female offspring ratio. Reproductively mature *Daphnia magna* were exposed to pyriproxyfen (155 or 310 pM) or arachidonic acid (1.0 or 4.0 µM) either alone or in combination and the number and sex of their neonates determined. (A) *D. magna* exposed to arachidonic acid alone, (B) *D.magna* exposed to pyriproxyfen alone, (C) *D.magna* exposed to 155pM of pyriproxyfen along with arachidonic acid and (D) *D.magna* exposed to 310 pM of pyriproxyfen along with arachidonic acid. An (a) indicates a significant difference in the total number of neonates produced, a (b) indicates a significant difference in the number of male neonates produced, and a (c) indicates a significant difference in the total number of female neonates produced as determined by one-way ANOVA followed by Dunnett's multiple comparison test (**= $p<0.01$, *** = $p<0.001$). Data are expressed as mean ± SEM.

Daphnids co-exposed to pyriproxyfen and AA show a decrease in the male/female sex ratio compared to daphnids only exposed to pyriproxyfen, primarily because of a significant increase in ratio of females in the co-exposed daphnids. The increased female neonate ratio also accounts for the increase in fecundity relative to daphnids exposed only to 310 pM pyriproxyfen, suggesting AA provides recovery or protection from pyriproxyfen induced repression of *Daphnia* reproduction (Ginjupalli and Baldwin, 2013) and male producing effects. However, at 155 pM pyriproxyfen, AA reduced male production and increased female production but without an overall increase in reproduction. In summary, AA altered the male/female sex ratio in pyriproxyfen-exposed daphnids primarily but not exclusively due to an increase in ratio of female neonates produced.

3.4.4 Influence of algal diet on reproduction:

Because AA enrichment resulted in changes in the male/female sex ratios following pyriproxyfen exposure and increased fecundity at a concentration of pyriproxyfen (310 pM) that represses overall reproduction, we investigated whether an algal diet low in AA represses reproduction in *D. magna. D. magna* were acclimatized to new dietary conditions without fish food and with different algae species that vary in AA concentration (*N. oculata*-high AA, *P. subcapitata*-moderately high AA*, C. vulgaris*-low AA concentrations) for three full generations. The number of neonates produced by adult fourth generation *D. magna* was quantified. A *C. vulgaris* diet, which is poor in AA,

resulted in a significant decrease in the number of offspring/adult compared to the two diets richer in AA (*N. oculata* and *P. subcapitata*) (Figure. 3.3).

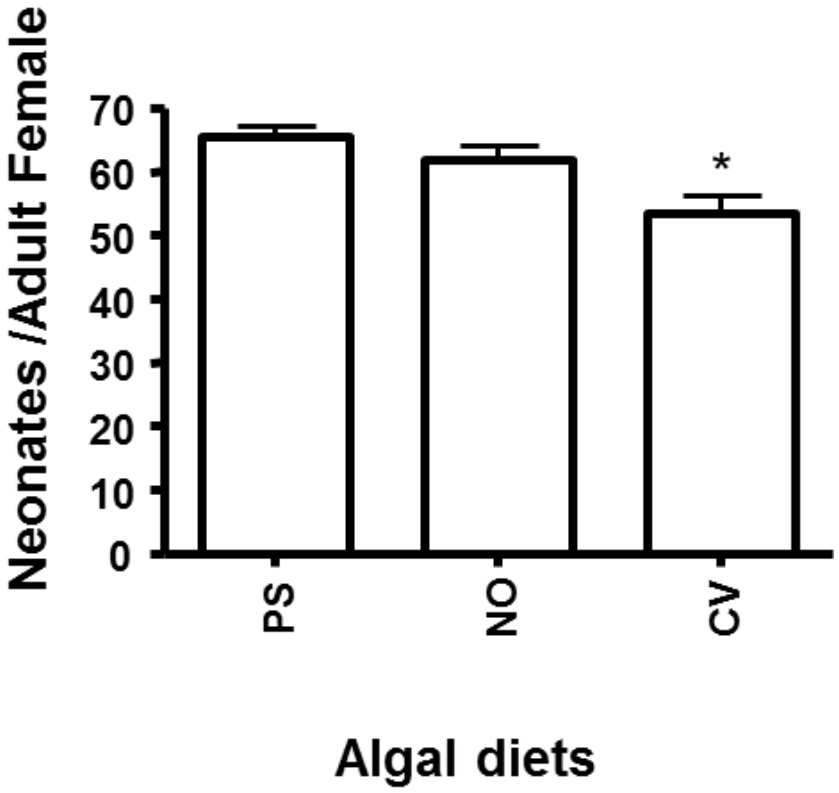


Figure. 3.3: Influence of algal diet with varying levels of arachidonic acid on *Daphnia* reproduction. *Daphnia magna* were acclimatized to algal diets for four generations. Influence of diets that differ in AA content was quantified based on total neonates produced in reproductively mature adults. An (a) indicates a significant difference in the total number of neonates produced as determined by one-way ANOVA followed by Dunnett's multiple comparison test (* = $p<0.05$). Data are expressed as mean ± SEM.

3.4.5 Influence of AA enrichment of an AA poor diet on fecundity and neonatal sex ratios:

Therefore, we examined the influence of AA supplementation on fecundity and male production with a *C. vulgaris* diet. Daphnids fed a *C. vulgaris* diet for four generations were exposed to pyriproxyfen, AA, or pyriproxyfen and AA. AA increased overall fecundity in a dose-dependent manner (Figure. 3.4) with or without pyriproxyfen present (52-95%) (Figure.3.4AB). AA also increased the number of males produced in a dose-dependent manner (45%) when co-treated with pyriproxyfen, but not the number of females as observed following the half *P. subcapitata* diet (Figure.3. 2).

Concurrently, a diet switch experiment was performed in which *D. magna* fed a moderately AA rich diet of *P. subcapitata* until 9-d old were switched to the AA poor *C. vulgaris* diet at the onset of the pyriproxyfen and AA exposures. Once again AA increased fecundity in the AA only and pyriproxyfen + AA groups in a dose-dependent manner (55% and 90%, respectively) (Figure. 3.4CD). However, during the diet switch experiments female production was significantly altered in the co-exposed group, but not male production. Thus, the diet switch experiment in which daphnids are exposed to *P. subcapitata* as neonates and *C. vulgaris* as adults is consistent with the previous experiment with *P. subcapitata* (Figure. 3.2), but not the experiment following acclimation to *C. vulgaris* (Figure.3. 4B), suggesting dietary AA sequestration following *P. subcapitata* feeding.

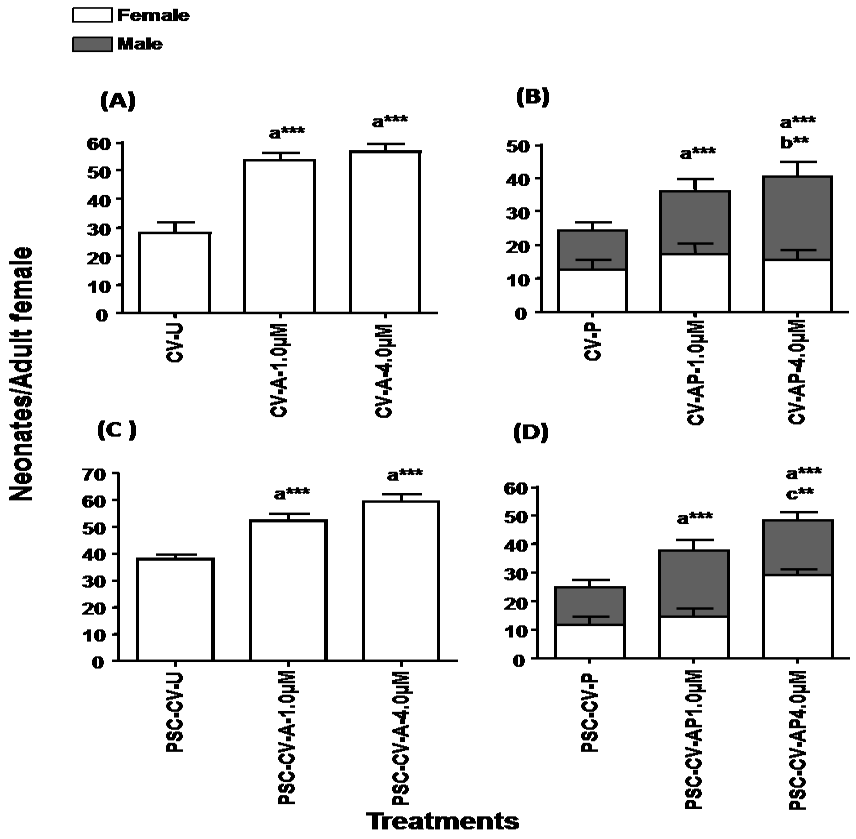


Figure. 3.4: Reproductive effects of supplementing an arachidonic acid poor diet (*Chlorella vulgaris*) with AA in diet-acclimatized and diet-switched daphnids. Reproductively mature *Daphnia magna* were exposed to 155 pM pyriproxyfen or 1.0µM or 4.0µM AA alone or in combination. Production of male and female offsprings was quantified (A) *D. magna* acclimatized to *C.vulgaris* for four generations and exposed to AA. (B) *D. magna* acclimatized to *C.vulgaris* for four generations and exposed to pyriproxyfen or a combination of pyriproxyfen and AA. (C) *D. magna* cultured with *P. subcapitata* and diet-switched to a *C.vulgaris* supplemented with 1.0µM or 4.0µM AA at 10 d old (D) *D. magna* cultured with *P. subcapitata* and diet-switched to a *C.vulgaris* and supplemented with 0, 1.0 or 4.0µM AA during pyriproxyfen-treatment at 10 d old. (CV) *Chlorella vulgaris*; (U) Untreated; (A) Arachidonic acid; (P) Pyriproxyfen-155pM (PSC) *Pseudokirchneriella subcapitata;* (PSC-CV-U) *P.subcapitata* switched to *C.vulgaris* untreated; (PSC-CV-A) *P.subcapitata* switched to *C.vulgaris* with arachidonic acid; (PSC-CV-P) *P.subcapitata* switched to *C.vulgaris* with pyriproxyfen; (PSC-CV-AP) *P.subcapitata* switched to *C.vulgaris* with arachidonic acid and pyriproxyfen. An (a) indicates a significant difference in the total number of neonates produced, a (b) indicates a significant difference in the number of male neonates produced, and a (c) indicates a significant difference in the total number of female neonates produced as determined by one-way ANOVA followed by Dunnett's multiple comparison test (** = $p<0.01$, *** = $p<0.001$). Data are expressed as mean ± SEM.

3.5 Discussion

Arachidonic acid enhances reproduction in dietary-restricted *Daphnia magna.* AA also increases female/male sex ratios in the presence of the JHA pesticide, pyriproxyfen. *Daphnia* accumulate AA from their phytoplankton diet and retain it in the ovaries (von Elert, 2002; Taipale et al., 2011) and eggs (Wacker and Martin-Creuzburg, 2007). This retention of AA is probably crucial in their reproductive physiology, "especially under restrictive or fluctuating dietary conditions" as AA supplementation of an abundant diet of *P. subcapitata* did not increase reproduction or alter pyriproxyfen-mediated male production. However, AA supplementation of an AA limited diet increased fecundity.

P. subcapitata is a typical laboratory daphnid diet and AA supplementation significantly improves reproduction in only a restricted *P. subcapitata* diet. *C. vulgaris* lack AA and daphnids fed a diet of *C. vulgaris* show significantly lower fecundity even, when fed 6 x 10^6 cells/adult (Figure.3.3). Supplementation of AA to daphnids cultured for four generations on a *C. vulgaris* diet significantly improved fecundity (Figure.3. 4AB) with or without pyriproxyfen present. In fact, the overall increase in fecundity was nearly the same with or without pyriproxyfen (60 to 90% Figure. 3.4AB). *N. oculata* and *P. subcapitata* algal diets with significantly greater AA concentrations (especially *N. oculata*) had nearly equivalent fecundity (Roncarati et al., 2004). This suggests that most diets contain the necessary AA to support reproduction, but restricted diets or a diet rich in *C. vulgaris* may not contain the necessary AA to support maximal reproduction.

Diet of mothers affects the fitness of neonates in *Daphnia longispina*. Neonates born to mothers that were on *Rhodomonas* a diet high in nutrients showed a greater

fitness and enhanced reproduction parameters compared to the neonates born to mothers on a *Microcystis* diet that is poor in nutrients (Brett, 1993). In addition, under natural conditions, the herbivorous zooplankton and phytoplankton interactions play crucial roles in dictating the algal succession. Because, *Daphnia* exert strong grazing pressure on phytoplankton as primary consumers of algae and also by immobilizing mineral nutrients mainly, phosphorus within their biomass (Rothhaupt, 1997). The changes in abundance and composition of algal growth depend up on variations in the photoperiods, climate and nutrient availability. Frequent short-term external perturbations such as changes in the outflows might also induce allogeneic shifts in algal succession. (Hoyer et al., 2009). In addition to the seasonal changes, the zooplankton herbivores can also influence population dynamics and dominance of certain algal species (Burkepile and Hay, 2010).

Therefore, a change in the diet depending on the season in nature appears to influence the reproductive and fitness traits of *Daphnia* that primarily depend on algae. Our diets switch experiments might be comparable to the effects of algal succession from nutrient rich to nutrient poor diets in nature and our results might as well be applied to understand the influence of algal succession on *Daphnia* under natural conditions, where daphnid populations face seasonal changes in availability and composition of diet due to algal succession that affects their survival and fecundity.

The diet switch experiments in which daphnids were switched at reproductive age from *P. subcapitata* to *C. vulgaris* did not produce an identical phenotype to the *C. vulgaris* only fed group. In the diet switch experiment, AA increased fecundity 55%, which is close to the 60-90% fecundity increases measured earlier. Typically, following

pyriproxyfen-exposure, increased female production is the primary driver of increased fecundity under a *P. subcapitata* diet; increased male production is the primary driver of increased fecundity under a *C. vulgaris* diet (Figure. 3.2 and 3.4). AA is one of few PUFAs retained (Goulden and Place, 1993; Taipale et al., 2011), and we hypothesize that its retention allows daphnids to acclimate to specific environmental and dietary challenges in a changing pond ecosystem. In turn, during the diet switch experiment, pyriproxyfen-exposure increased female production similar to daphnids fed *P. subcapitata*. We hypothesize that the retention of AA during early feedings with *P. subcapitata* is crucial in the differences observed in sex ratios as AA is a component of a *P. subcapitata* diet, but not a *C. vulgaris* diet. Thus, the retained AA provided some protection from male production.

Our studies also demonstrate the importance of a healthy diet in a proper response to toxicant exposure. AA supplementation clearly helped support reproduction in the presence of the JHA pesticide, pyriproxyfen. Pyriproxyfen significantly reduces fecundity (Figure. 3. 2) (Ginjupalli and Baldwin, 2013). Supplementation (1.0 or 4.0µM) of *C. vulgaris* or half *P. subcapitata* with AA prompted a recovery caused by the pyriproxyfen-induced (155 pM or 310 pM pyriproxyfen) drop in fecundity (Figure. 2BD, Figure. 4BD). AA supplementation overcomes this repression of fecundity when diets are restricted either by the number of algae (half *P. subcapitata*) or the source of algae (*C. vulgaris*). Diets are not restricted during environmental toxicology tests; however, algal restricted diets may be common in ponds, especially prior to summer algal blooms. Furthermore, algal diets may not be ideal because of the composition of the algae in the

pond or lake (Ahlgren et al., 1990;Alam et al., 2001; Salmaso, 2010; Hartwich et al., 2012). Therefore, pyriproxyfen may have greater effects than expected under some specific adverse conditions depending on the algae present during that season. It is possible that AA works directly to overcome adverse effects of pyriproxyfen on fecundity, or AA alters sex through disruptive regulation of the sexual cycle such as the production of doublesex (Kato et al., 2011a). However, we consider it more likely that AA is a key PUFA in reproduction and in turn increases fecundity under many adverse circumstances including exposure to pyriproxyfen. The adverse effects of AA on sex ratios depending on diet are interesting and may be caused by one or more pathways modulated by AA.

The beneficial effects observed due to AA supplementation of AA poor diets appears to be independent of general energetics of *Daphnia*. Because, *Daphnia* primarily invest the dietary PUFAs for growth and reproduction (Brett and Muller-Navarra, 1997). *Daphnia* normally depend on energy rich saturated fatty acids to meet their for energy needs (Brett and Muller-Navarra, 1997). Most of the physiological needs for PUFAs in *Daphnia* are met through dietary sources (Goulden and Place, 1993). In addition dietary PUFAs have more important roles, such as eicosanoid synthesis and immune functions (Miller et al., 1994). Therefore, they can be considered as precious resource required for overall growth and reproduction. Therefore, the beneficial effects observed in our study could be due to the involvement of AA directly or through formation of related metabolites, such as prostaglandins.

The inhibition of HR97 receptors by AA and the activation of pyriproxyfen by HR97g in vitro (Appendix.B3) are interesting observations, which provide a possible mechanistic pathway for this effect. This makes HR97g and the other HR97 receptors potential targets for RNAi experiments. RNAi has been developed for embryonic *D. magna* (Kato et al., 2011a; Kato et al., 2011b). However, HR97 receptors are expressed in adults (Appendix.B1 and Appendix.B.2) (Ginjupalli et al., 2011) and the dilution of RNAi may not repress HR97 receptors 14 d following RNAi exposure. In addition, the concentration of pyriproxyfen needed to activate HR97g (> 3 μM) may be outside reasonable physiological ranges. The concentration of AA needed to activate HR97 receptors is quite high (25-50 μM); however, it has been reported that AA concentrations can reach up to 100 μM under inflammatory conditions in humans (Brash, 2001). We consider it unlikely that pyriproxyfen works through HR97g as recently the JHA receptor in daphnids was discovered independently by two groups (LeBlanc et al., 2013; Miyakawa et al., 2013). Therefore, as mentioned above, we consider it much more likely that AA is retained in the ovaries as a key PUFA in reproduction and in turn increases fecundity under many circumstances including adverse circumstances such as exposure to pyriproxyfen. Further, we hypothesize that AA is retained so that it is maintained at or above threshold concentrations in the ovaries.

Fatty acid composition of *Daphnia* matches their dietary fatty acid composition (Brett et al., 2006). AA is specifically retained in the ovaries of *Daphnia* (Goulden and Place, 1993). Several investigators have hypothesized that AA is crucial in daphnid reproduction. In addition, previous studies demonstrated that dietary AA concentrations

enhance percent females per spawn, number of spawnings, eggs per female and promotes egg development in marine shrimp, *P. monodon* (Coman et al., 2011). However, a recent study indicated that AA is not crucial for reproduction of *Daphnia pulex* compared to EPA (Ravet et al., 2012). AA was supplemented in this study; however, AA concentrations were not completely restricted in the controls. Taken together our data, plus the data from the literature may indicate that there is a threshold AA concentration in the daphnid ovary and a drop in ovarian AA level below the threshold concentrations must occur for perturbations in reproduction to be evident. Based on our results, we hypothesize that the algal diet used and the amount of algae fed to each adult could significantly alter the results. Ravet et al. (2012) indicate surprise with their results and also suggest that a different diet may produce significantly different results.

In summary, AA supplementation enhanced fecundity compared to similarly treated daphnids during a restricted diet. In addition, AA supplementation enhanced fecundity and female/male ratios under the stress of JHA exposure during restricted diets, indicating the adverse hormonal effects of the toxicants were mitigated by AA. Overall, pyriproxyfen is more toxic when dietary resources were limited as compared to a diet complete with supplemented AA. This indicates that diet is crucial in recovery or acclimation to some toxicants as AA supplementation significantly improved fecundity in control daphnids and daphnids exposed to the JHA pesticide, pyriproxyfen.

3.6 References

Ahlgren, G., Lundstedt, L., Brett, M., Forsberg, C., 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. J Plankton Res 12, 809-818

Alam, M.G.M., Jahan, N., Thalib, L., Wei, B., Maekawa, T., 2001. Effects of environmental factors on the seasonally change of phytoplankton populations in a closed freshwater pond. Environ Int 27, 363-371.

(ASTM), American Society for Testing and Materials, 1993. Guide for conducting renewal life-cycle toxicity tests with *Daphnia magna*. ASTM, Standards on Aquatic Toxicology and Hazard Evaluation., Philadelphia, PA, pp. 151-167.

Anderson, B., Jenkins, J., 1942. A time study of events in the life span of *Daphnia Magna*. Biol Bull 83, 260-272.

Baldwin, W.S., Bailey, R., Long, K.E., Klaine, S., 2001. Incomplete ecdysis is an indicator of ecdysteroid exposure in *Daphnia magna*. Environ Toxicol Chem. 20, 1564-1569.

Baldwin, W.S., Graham, S.E., Shea, D., LeBlanc, G.A., 1997. Metabolic androgenization of female *Daphnia magna* by the xenoestrogen 4-nonylphenol. Environ Toxico Chem. 16, 1905-1911.

Bec, A., Desvilettes, C., Vera, A., Lemarchand, C., Fontvieille, D., Bourdier, G., 2003. Nutritional quality of a freshwater heterotrophic flagellate: Trophic upgrading of its microalgal diet for *Daphnia hyalina*. Aquat Microb Ecol. 32, 203-207.

Brash, A.R., 2001. Arachidonic acid as a bioactive molecule. J Clin Invest 107, 1339-1345.

Brett, M.T., 1993. Resource quality effects on *Daphnia-longispina* offspring fitness. J Plankton Res 15, 403-412.

Brett, M.T., Mueller-Navarra, D., Ballantyne, A., Ravet, J., Goldman, C., 2006. *Daphnia* fatty acid composition reflects that of their diet. Limnol Oceanogr 51, 2428-2437.

Brett, M.T., Muller-Navarra, D.C., 1997. The role of highly unsaturated fatty acids in aquatic food web processes. Freshwater Biol 38, 483-499.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151, 315-331.

Burkepile, D.E., Hay, M.E., 2010. Impact of herbivore identity on algal succession and coral growth on a caribbean reef. Plos One 5, e8963

Büyükgüzel, E., Tunaz, H., Stanley, D., Büyükgüzel, K., 2011. The influence of chronic eicosanoid biosynthesis inhibition on life history of the greater waxmoth, *Galleria mellonella* and its ectoparasitoid, *Bracon hebetor*. J Insect Physiol 57, 501-507.

Carpenter, S., Kitchell, J., Hodgson, J., Cochran, P., Elser, J., Elser, M., Lodge, D., Kretchmer, D., He, X., Von, E., 1987. Regulation of lake primary productivity by food web structure. Ecology 68 1863-1876.

Chang, K.J., Kim, J.W., Im, W.B., Kang, H.M., Kwon, H.B., 1997. Differential effects of gonadotropin and orthovanadate on oocyte maturation, ovulation, and prostaglandin synthesis by Rana ovarian follicles in vitro. J Exp Zool 277, 155-165.

Coman, G.J., Arnold, S.J., Barclay, M., Smith, D.M., 2011. Effect of arachidonic acid supplementation on reproductive performance of tank-domesticated *Penaeus monodon*. Aquaculture Nutrition 17, 141-151.

Cox, R., Calsbeek, R., 2010. Severe costs of reproduction persist in anolis lizards despite the evolution of a single-egg clutch. Evolution 64, 1-10.

Deng, H., Lynch, M., 1996. Change of genetic architecture in response to sex. Genetics 143 203-212.

Gaedke, U., Straile, D., 1998. Daphnids: Keystone species for the pelagic food web structure and energy flow - A body size-related analysis linking seasonal changes at the population and ecosystem levels. Advance Limnol 53, 587-610.

Ginjupalli, G., Baldwin, W., 2013. The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on *Daphnia magna* reproduction. Chemosphere 92, 1260-1266.

Ginjupalli, G., Y, L., Baldwin, W., 2011. "The novel *Daphnia* nuclear receptor, HR97g" is activated by juvenile hormone analogs. Society of Toxicology Annual Meeting. The Toxicologist#348, Washington,DC.

Goulden, C.E., Place, A.R., 1993. Lipid accumulation and allocation in Daphnid Cladocera. Bull Marine Sci Volume 53, 106-114.

Guedes, A.C., Amaro, H., Barbosa, C., Pereira, R., Malcata, F., 2011. Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on

eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses. Food Res International 44, 2721-2729.

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog Lipid Res 45, 160-186.

Hartwich, M., Straile, D., Gaedke, U., Wacker, A., 2012. Use of ciliate and phytoplankton taxonomic composition for the estimation of eicosapentaenoic acid concentration in lakes. Freshwater Biol 57, 1385-1398.

Heckmann, L.H., Sibly, R.M., Timmermans, M., Callaghan, A., 2008. Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. Front Zool 5:11.

Horner, M., Pardee, K., Liu, S., King-Jones, K., Lajoie, G., Edwards, A., Krause, H., Thummel, C., 2009. The *Drosophila* DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis. Genes Dev 23, 2711-2716.

Hoyer, A.B., Moreno-Ostos, E., Vidal, J., Blanco, J.M., Palomino-Torres, R.L., Basanta, A., Escot, C., Rueda, F.J., 2009. The influence of external perturbations on the functional composition of phytoplankton in a Mediterranean reservoir. Hydrobiologia 636, 49-64.

Karimullina, E., Li, Y., Ginjupalli, G.K., Baldwin, W.S., 2012. *Daphnia* HR96 is a promiscuous xenobiotic and endobiotic nuclear receptor. Aquat Toxicol 116, 69-78.

Kato, Y., Kobayashi, K., Watanabe, H., Iguchi, T., 2011a. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a doublesex gene in the sex-determining pathway. PLoS Genetics 7, e1001345.

Kato, Y., Shiga, Y., Kobayashi, K., Tokishita, S., Yamagata, H., Iguchi, T., Watanabe, H., 2011b. Development of an RNA interference method in the cladoceran crustacean *Daphnia magna*. Dev Genes Evol 220, 337-345.

King-Jones, K., Horner, M.A., Lam, G., Thummel, C.S., 2006. The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. Cell Metab 4, 37-48.

Kleiven, O.T., Larsson, P., Hobæk, A., 1992. Sexual reproduction in *Daphnia magna* requires three stimuli. Oikos 65, 197-206.

LeBlanc, G.A., Wang, Y.H., Holmes, C.N., Kwon, G., Medlock, E.K., 2013. A transgenerational endocrine signaling pathway in crustacea. Plos One 8, e61715.

Meester, L., Doorslaer, W., Geerts, A., Orsini, L., Stoks, R., 2011. Thermal Genetic Adaptation in the water flea *Daphnia* and its impact: An evolving metacommunity approach. Integ Comp Biol 51, 703–718

Michael, B., Dorthe, M., Muller, N., 1997. The role of highly unsaturated fatty acids in aquatic food web processes. Freshwater Biol 38, 483–499.

Miller, W., Geeene, J., Shiroyama, T., 1978. The *Selenastrum capricornutum* printz algal assay bottle test. Experimental design,application, and data interpretation protocol. Environmental Reserach Laboratory, Corvalis, Oregon, 97330, pp. 11-26.

Miller, J.S., Nguyen, T., Stanleysamuelson, D.W., 1994. Eicosanoids mediate insect nodulation responses to bacterial-infections. Proceedings of the Nat Acad Sci of the United States of America. 91, 12418-12422.

Miyakawa, H., Toyota, K., Hirakawa, I., Ogino, Y., Miyagawa, S., Oda, S., Tatarazako, N., Miura, T., Colbourne, J.K., Iguchi, T., 2013. A mutation in the receptor methoprene-tolerant alters juvenile hormone response in insects and crustaceans. Nature Commun 4.

Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., Lefkowith, J.B., 1986. Arachidonic-acid metabolism. Ann Rev Biochem 55, 69-102.

Oda, S., Tatarazako, N., Watanabe, H., Morita, M., Iguchi, T., 2005. Production of male neonates in *Daphnia magna* (*Cladocera, Crustacea*) exposed to juvenile hormones and their analogs. Chemosphere 61, 1168-1174.

Olmstead, A.W., LeBlanc, G.A., 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. J Exp Zool 293, 736-739.

Olmstead, A.W., LeBlanc, G.A., 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environ Health Perspect 111, 919-924.

Ravet, J., Persson, J., Brett, M.T., 2012. Threshold dietary polyunsaturated fatty acid concentrations for *Daphnia pulex* growth and reproduction. Inland Waters 2, 199-209.

Ravid, T., Tietz, A., Khayat, M., Boehm, E., Michelis, R., Lubzens, E., 1999. Lipid accumulation in the ovaries of a marine shrimp *Penaeus semisulcatus* (De Haan). J Exp Biol 202, 1819-1829.

Rothhaupt, K.O., 1997. Grazing and nutrient influences of *Daphnia* and *Eudiaptomus* on phytoplankton in laboratory microcosms. J Plankton Res 19, 125-139.

Roncarati, A., Meluzzi, A., Acciarri, S., Tallarico, N., Melotti, P., 2004. Fatty acid composition of different microalgae strains (*Nannochloropsis* sp., *Nannochloropsis oculata* (Droop) Hibberd, *Nannochloris atomus* Butcher and *Isochrysis* sp.) according to the culture phase and the carbon dioxide concentration. J World Aquaculture Society 35, 401-411.

Salmaso, N., 2010. Long-term phytoplankton community changes in a deep subalpine lake: responses to nutrient availability and climatic fluctuations. Freshwater Biol 55, 825-846.

Sieber, M., Thummel, C., 2012. Coordination of triacylglycerol and cholesterol homeostasis by DHR96 and the *Drosophila* LipA homolog magro. Cell Metab 15, 122-127.

Sieber, M.H., Thummel, C.S., 2009. The DHR96 nuclear receptor controls triacylglycerol homeostasis in *Drosophila*. Cell Metab 10, 481-490.

Smith, A., Acharya, K., Jack, J., 2009. Overcrowding, food and phosphorus limitation effects on ephippia production and population dynamics in the invasive species *Daphnia lumholtzi*. Hydrobiologia 618, 47-56.

Stanley, D., 2006. Prostaglandins and other eicosanoids in insects: Biological significance. Annu Rev Entomol, pp. 25-44.

Stanleysamuelson, D.W., Loher, W., 1986. Prostaglandins in insect reproduction. Ann Entomol Soc Am 79, 841-853.

Stephan, C.E., 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, Duluth, MN.

Taipale, S.J., Kainz, M.J., Brett, M.T., 2011. Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids in *Daphnia*. Oikos (Epub ahead of print: doi: 10.1111/j.1600-0706.2011.19415.x).

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., Iguchi, T., 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53, 827–833.

Thomson, S.A., Baldwin, W.S., Wang, Y.H., Kwon, G., LeBlanc, G.A., 2009. Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. BMC Genomics 10, 500.

Verreth, J., Coppoolse, J., Segner, H., 1994. The effect of low HUFA- and high HUFA-enriched Artemia, fed at different feeding levels, on growth, survival, tissue fatty acids and liver histology of *Clarias gariepinus* larvae. Aquaculture 126, 137-150.

von Elert, E., 2002. Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. Limnol Oceanogr 47, 1764-1773.

Wacker, A., Martin-Creuzburg, D., 2007. Allocation of essential lipids in *Daphnia magna* during exposure to poor food quality. Funct Ecol 21, 738-747.

Wang, H.Y., Olmstead, A.W., Li, H., LeBlanc, G.A., 2005. The screening of chemicals for juvenoid-related endocrine activity using the water flea *Daphnia magna*. Aquat Toxicol 74, 193-204.

CHAPTER FOUR

REPRODUCTIVE AND DEVELOPMENTAL DEFECTS IN *DAPHNIA MAGNA* CAUSED BY IBUPROFEN EXPOSURE ARE DIET-DEPENDENT AND MITIGATED BY ARACHIDONIC ACID

4.1 ABSTRACT

There is growing concern that the increase in pharmaceutical chemicals in our wastewater and surface waters that degrade our ecosystem and having significant effects on aquatic biota. *D. magna* are a key aquatic indicator species often used to determine the potential for chemicals to perturb the environment and more recently to estimate the mechanism of action of toxic chemicals on aquatic invertebrates. We previously showed that arachidonic acid (AA), a dietary ω-6 fatty acid is important for reproduction and showed that it enhances reproduction in *D.magna* under restrictive diets (Chapter-3). However, the mechanisms associated with the beneficial role of AA in supporting reproduction under AA low diets are not clearly understood. We hypothesized that AA metabolism is crucial for its role in enhancing reproduction. Therefore, we used ibuprofen, a commonly used non-steroidal anti-inflammatory drug (NSAID) to inhibit synthesis of AA metabolites such as the eicosanoids. Our data indicate that ibuprofen reduces fecundity in an AA-dependent manner as AA supplementation reverses the adverse effects of ibuprofen. In addition, we observed that ibuprofen induced developmental abnormalities at 12 and 24 mg L^{-1}, and these phenotypes were in part reversed also by AA. This study is helpful in providing a basic understanding of how ibuprofen acts in *D. magna* and the importance of AA in the diet for proper development and reproduction.

4.2 Introduction

Daphnia are freshwater micro crustaceans used as aquatic indicator species (Carpenter et al., 1987). They are primary consumers of algae and a food source for larger invertebrates and small fish (Gaedke and Straile, 1998).Therefore, healthy reproduction of *Daphnia* contributes to the sustenance of the ecosystem by providing trophic transfer of key essential nutrients (Taipale et al., 2011).

Polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) are key components of a healthy diet and proper reproduction in many species (Sargent et al., 1999). For example, diets rich in the ω-3 fatty acids, EPA and DHA increase reproduction in the marine copepod, *Acartia tonsa* (Dana) (Støttrup and Jensen, 1990). AA, EPA, and DHA all enhance reproduction in fish (Koven et al., 1992; Koven et al., 2001; Phelps, 2010; Zuo et al., 2012). Eggs containing higher concentrations of DHA showed higher fertilization, hatching and larval survival rates in freshwater fish *C.undecinmalis* (Lee, 2003; Yanes-Roca et al., 2009). In *Daphnia* species, EPA and AA are specifically retained in the ovaries (Ahlgren et al., 1990; Goulden and Place, 1993; Bec et al., 2003; Wacker and Martin-Creuzburg, 2007; Taipale et al., 2011). Both of these polyunsaturated fatty acids have recently been shown to increase reproduction (Ravet et al., 2012; Ginjupalli and Baldwin, submitted), although AA only did so under resource limited conditions (Ginjupalli and Baldwin, submitted).

Arachidonic acid (AA) is an ω-6 dietary fatty acid that is specifically retained in *Daphnia* from their algal diet and in turn stored in the ovaries of *Daphnia* (Ahlgren et al.,

1990; Bec et al., 2003; Taipale et al., 2011). Though, *Daphnia* have at least one cyclooxygenase (COX) gene (NCBI accession number EFX85708.1) (Heckmann, 2008), and are capable of synthesizing AA from the bodily sources, they primarily depend on dietary fatty acids to meet the physiological demands. Fatty acid synthesis rates from tissue sources are as low as 0.16% to 1.6% depending on the quality of diet (Goulden and Place, 1993). Therefore, we hypothesized that AA is important for reproduction and development. Previous research (Chapter 3) indicates that AA enhances fecundity in *D. magna* under resource limited conditions and partially protects against pyriproxyfen-induced male production (Ginjupalli and Baldwin, submitted). Diets that are low in AA, as well as EPA and DHA (*C. vulgaris*) compared to diets that are moderately rich in these polyunsaturated fatty acids (*P. subcapitata* and *N.oculata*) improve the growth and reproduction of *Daphnia* (Brett and Muller-Navarra, 1997; Ravet et al., 2012; Ginjupalli and Baldwin, submitted) . Diets rich in AA contribute to improved reproduction in *Daphnia* and can be protective against toxicity (Ginjupalli and Baldwin, submitted). Evidence indicates that AA can help daphnids recover from toxicants causing reproductive distress (Ginjupalli and Baldwin, submitted).

There is a growing concern with regards to the accumulation of widely used pharmaceuticals in the environment with some pharmaceuticals detected at concentrations ranging from ng - mg L^{-1}quantities in aquatic environment (Kolpin et al., 2002; Kosjek et al., 2005). Ibuprofen is a commonly used antipyretic and anti-inflammatory compound that suppresses the synthesis of prostaglandins and eicosanoids from AA through inhibition of cyclooxygenases (Bancos et al., 2009). *Daphnia* contain at

least one cyclooxygenase (NCBI accession number EFX85708.1) (Heckmann, 2008), and AA metabolism pathways are enhanced in *Daphnia pulex* relative to other species (Colbourne et al., 2011). Therefore, we hypothesize that a proper diet or a diet rich in AA can help daphnids recover from the ibuprofen induced toxicity.

Ibuprofen is excreted either unchanged or as a mixture of metabolites and can enter ground waters and drinking water as it is not completely eliminated by traditional waste water treatment processes (Doll and Frimmel, 2003). Ibuprofen is relatively common in the environment and found in 9.5 percent of surface waters tested (Kolpin et al., 2002). It has been found at 0.03 μg L^{-1} in major rivers in Korea (Kim et al., 2007), 0.1 μg L^{-1} in surface waters in South Whales of UK (Kasprzyk-Hordern et al., 2008) and 3.0 μg L^{-1} in effluents from wastewater treatment plants in Switzerland (Buser et al., 1999).

The frequency of occurence of ibuprofen in surface and effluent waters accounts for as high as > 80 % in major water bodies in Korea. Even though, the conventional methods of removal is not efficient in removing ibuprofen, WWTP that use granular activated carbon can remove up 99% of the ibuprofen (Kim et al., 2007). Due to several factors such as environmental oxidative degradation, influence of season and dilution in large bodies of water contribute to large variations in the measured concentrations of ibuprofen with conflicting data from the past (Buser et al., 1999). However, it is surprising to note that the measured concentrations of ibuprofen in the surface waters of Lake Michigan are as low as 32 ng L^{-1}, which might potentially be due to dilution in large body of water (Uslu et al., 2013).

Ibuprofen is toxic to invertebrates; albeit at concentrations much greater than those commonly found in surface waters. An important reason for investigating the effects of ibuprofen, even at levels greater than found in environmental systems, is that there are typically several pharmaceuticals released in the effluent of WWTPs and that the total concentrations are likely in the mg L^{-1} range. Ibuprofen decreases wet weight in the freshwater snail, *Planorbis carinatus,* in a 21 d assay with a lowest observed effect concentration (LOEC) value of 2.43 mg L^{-1} and a no observed effect concentration (NOEC) value of 1.02 mg L^{-1} (Pounds et al., 2008). Ibuprofen has a 48h immobilization EC50 of 72.6 mg L^{-1} and a 7 d reproduction NOEC value of 25 mg L^{-1} in the crustacean *Monia macrocopa*. The EC50 of ibuprofen for 48h immobilization in *D.magna* was 51.4 mg L^{-1} (Han et al., 2010) and LC50 was 132.6 mg L^{-1} (Han et al., 2006). Ibuprofen chronic exposure for 21 d in neonates showed a NOEC of 33.3 mg L^{-1} based on survival and a LOEC of 1.23 mg L^{-1} based on reproduction (Han et al., 2010). Fish appear to be more sensitive as in Japanese medaka (*Oryzias latipes*) ibuprofen induces vitellogenin production in male fish and adversely affects the hatching of eggs from parental exposures at 0.0001 mg L^{-1} ibuprofen (Han et al., 2010).

The purpose of this study is to determine the specific reproductive and developmental effects of ibuprofen on adult *D. magna,* and the potential role of AA in mitigating the toxicity of ibuprofen. Ibuprofen inhibits the metabolism of AA to prostaglandins by inhibiting cyclooxygenases (Mitchell et al., 1993; Bancos et al., 2009). Considering the physiological roles played by AA and its metabolites in survival and

reproduction in several different species, we hypothesized ibuprofen toxicity would in part be mitigated by AA by overcoming cyclooxygenase inhibition.

4.3 Materials and methods

4.3.1 Daphnia magna and algae culture:

Algae cultures of *P. subcapitata* (Aquatic Biosystems, Fort Collins, CO, USA) or *C. vulgaris* (Algae Depot, Eu Claire, WI USA) are cultured in our laboratory per EPA guidelines in prepared media and under continuous fluorescent light (Miller et al., 1978). *D. magna* are cultured under standard conditions of a 16:8 hour light cycle at 21^{o}C in an environmental chamber. *Daphnia* cultures are grown in reconstituted moderately hard water and fed either a diet of *P. subcapitata* or *C. vulgaris* at 6 million cells/adult daphnid/day. Stock cultures of *D. magna* were fed *P. subcapitata* supplemented with 0.25 mg dry weight of blended TETRAFIN fish flakes (catalog # 46798-16140; Tetra Holding Inc., VA) in a 50 µL aqueous suspension (Baldwin et al., 2001; Ginjupalli and Baldwin, 2013).

4.3.2 Adult reproduction following exposure to ibuprofen:

Prior to reproduction tests, daphnids were acclimatized to different feeding conditions to restrict their dietary source of AA. *C. vulgaris* has very low levels of AA and *P. subcapitata* has moderate levels of AA (Brown et al., 1997; Guschina and Harwood, 2006; Guedes et al., 2011). Therefore, for some tests daphnids were switched from their stock food cultures of *P. subcapitata* and fish food to either *P. subcapitata*

only (for one or two generations) or to *C. vulgaris* for three full generations before the reproductive tests were initiated.

To determine the potential of AA in protecting the *Daphnia* from ibuprofen toxicity and characterize the adverse reproductive effects of ibuprofen we treated reproductively mature, ten day old *D. magna* acclimatized to AA restrictive diets with 6-24 mg L^{-1} ibuprofen sodium salt (≥ 98% Sigma-Aldrich, St. Louis, MO USA), 1.0 μM AA (>99% purity, CAS Number 506-32-1, Sigma-Aldrich, St. Louis, MO USA), both, or neither (control) for 10 d. Reproduction was quantified following exposure for the next four broods or approximately 10 d (Wang et al., 2005; Ginjupalli and Baldwin, 2013). During the study, daphnids were individually housed in 40 mL (n=10) of culture medium and media changed every other day. All treatment groups and the control group received 0.00025% ethanol as AA was carried in ethanol.

4.3.3 Developmental defects induced by ibuprofen:

Developmental abnormalities such as aborted eggs (lethal), deformed secondary antenna or apical spine (non-lethal) were evaluated and quantified by visual inspection of the progeny using a dissecting microscope (American Optical-150W haloid cold light source). The developmental defects were further analyzed and confirmed at a greater magnification of 500 or 1000X obtained by scanning electron microscopy (TM3000; Tabletop Microscope, Tokyo, Japan) at the electron microscopy facility at Clemson University using 1 and 5 d old daphnids that were fixed in 10% formaldehyde, processed in hexamethyldisilazine (HMDS)(Sigma-Aldrich).

4.3.4 Statistics:

Statistical differences $p < 0.05$, $p < 0.01$ or $p < 0.001$ were determined by ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism Version 4.3 (GraphPad Software La Jolla CA, USA).

4.4 Results

4.4.1 Influence of arachidonic acid (AA) rich and poor diets on Daphnia reproduction

D. magna were provided a normal diet, acclimatized to diets without fish food supplements, or acclimatized to a low AA diet (*C.vulgaris*) to determine if ibuprofen showed differential toxicity depending on the diet. Ibuprofen was only toxic to daphnids fed *C. vulgaris* or early in their acclimation to only *P. subcapitata* without the fish food supplement clearly showing that ibuprofen toxicity is diet dependent (Figure.4. 1). Supplementation with 1.0 μM AA mitigated ibuprofen toxicity in the two treatments with access to lower amounts of AA (Figure. 4. 1BD), suggesting that ibuprofen toxicity is through alterations in AA metabolism.

Ibuprofen has no adverse effects on fecundity in *D. magna* fed *P. subcapitata* supplemented with fish food (our typical diet) or *P. subcapitata* without fish food for two generations prior to the toxicity test (Figure. 4. 1). Contrary to the beneficial effects observed with AA supplementation of a low AA diet (*C. vulgaris*) (Figure.4.1D), AA supplementation of moderately AA rich diet caused a dose-dependent drop in fecundity (Figure.4. 1A). We hypothesize that these daphnids received too much AA as AA is toxic when supplemented at about 8 mM (Ginjupalli and Baldwin, submitted). Overall, diet

significantly effects toxicity and AA can mitigate toxicity when daphnids are fed an AA poor diet.

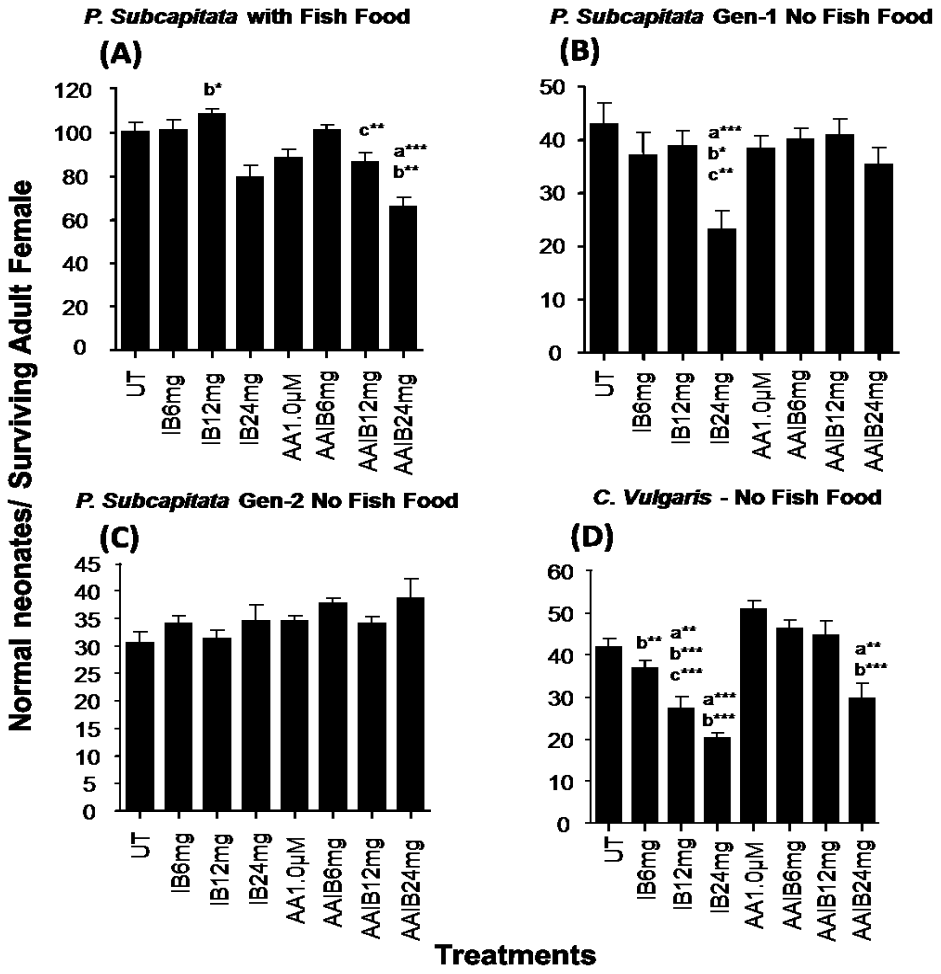


Figure. 4.1: Influence of diets rich and poor in AA and arachidonic acid (AA) exposures on *Daphnia* fecundity: Reproductively mature *D. magna* were acclimatized to different diets that vary in AA; and fecundity determined during ibuprofen, AA, or combination treatments. Stock *P. subcapitata* and fish food (A), acclimatized to *P. subcapitata* for one (B) or two generations (C) and *Chlorella vulgaris* (Low in AA) (D). (a) Indicates a significant difference from untreated daphnids, (b) indicates significant difference from AA treated daphnids, and (c) indicates a significant difference between IB and the corresponding IB+AA treatments as determined by one-way ANOVA followed by Tukey's multiple comparison test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Data are shown as mean ± SEM.

4.4.2 Developmental defects observed in neonates following ibuprofen exposure

Significant developmental abnormalities were observed during the reproduction tests. Visual inspection of the neonates indicated that these developmental abnormalities manifest themselves as either aborted (dead) neonates, dead upon release of the brood, or as non-lethal abnormalities. Non-lethal developmental defects include reduced digits such as shorter length of the digits, shortened flagellum on secondary antenna, or curved apical spines (Figure.4. 2). For better resolution of the structural details the defective *D. magna* were analyzed by scanning electron microscopy providing significant detail of modifications in secondary antenna and flagellum (Figure. 4. 2).

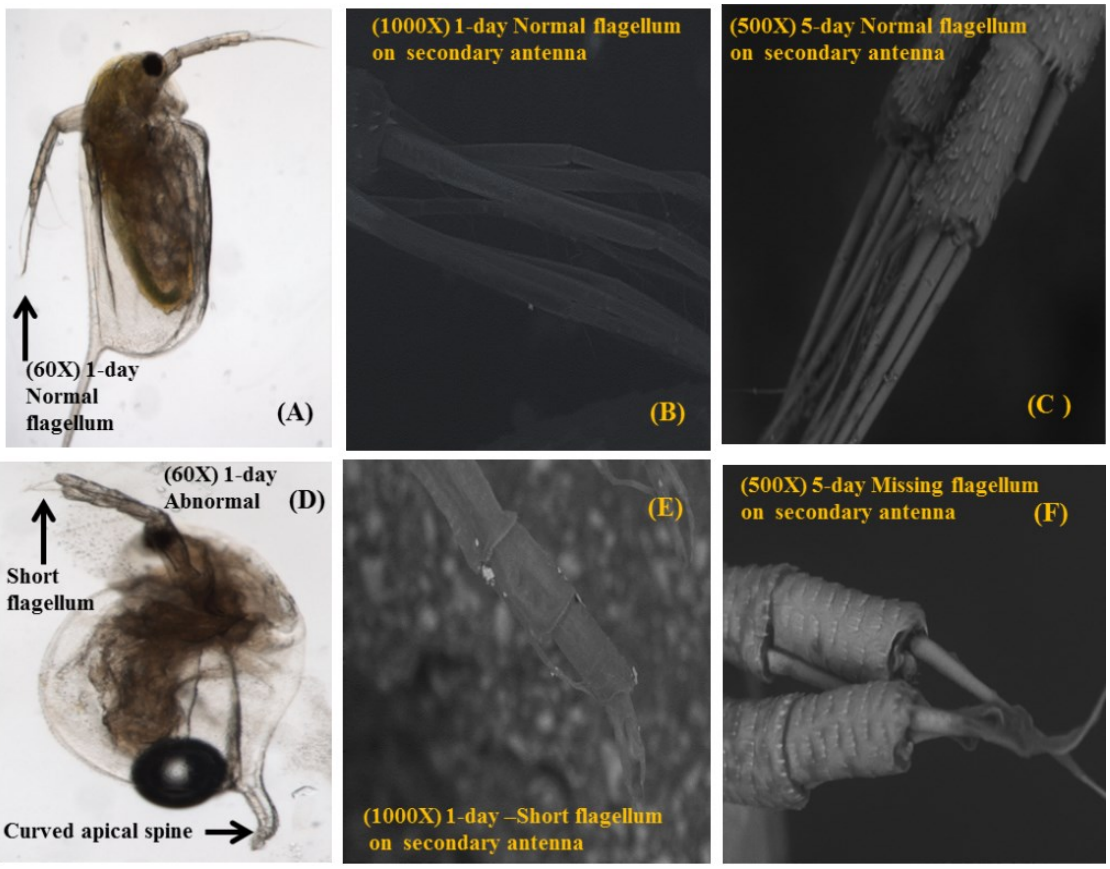


Figure. 4.2: Developmental toxicity of ibuprofen observed following a *C. vulgaris* diet: Ibuprofen exposure at 12 mg/L and 24.0 mg/L induced developmental defects in the secondary antenna. Growth of flagellum on secondary antenna was obliterated or shortened as determined by the scanning electron microscope TM-3000. A 1-d old normal neonate showing normal flagellum at 60x magnification/ NIKONSMZ1500-Sereo Microscope (A), a 1-d old normal neonate showing normal length of digits and flagellum on secondary antenna at 1000x magnification/ TM3000 scanning electron microscope (B), a 5-d old normal neonate showing normal length of digit and terminal flagellum on secondary antenna at 500x magnification/TM3000 scanning electron microscope (C), a 1-d old abnormal neonate showing short flagellum, short digits on secondary antenna and curved apical spine at 60x magnification/NIKONSMZ1500-Sereo Microscope (D), a 1-d old abnormal neonate showing short digits and short flagellum on secondary antenna at 1000x magnification/ TM3000 scanning electron microscope (E), a 5-d old normal neonate showing short digits and two missing terminal flagellum on secondary antenna at 500x magnification/TM3000 scanning electron microscope(F).

Quantification of the lethal and non-lethal adverse effects of ibuprofen on daphnid development demonstrated that these effects are more pronounced in the daphnids fed a low AA diet (*C. vulgaris*), or recently switched to a *P. subcapitata* diet without fish food as a supplement. The total number of abnormalities (lethal and non-lethal) was typically dose-dependent following ibuprofen exposure (Figure.4. 3). AA supplementation often reduced the developmental defects caused by ibuprofen; however, rarely was this reduction statistically significant with the exception of AA provided with ibuprofen in *D. magna* fed *C. vulgaris*. Because reproduction varied considerably between diets and was much greater in the fish food supplemented daphnids, percent abnormalities is also provided as Appendix.D.1

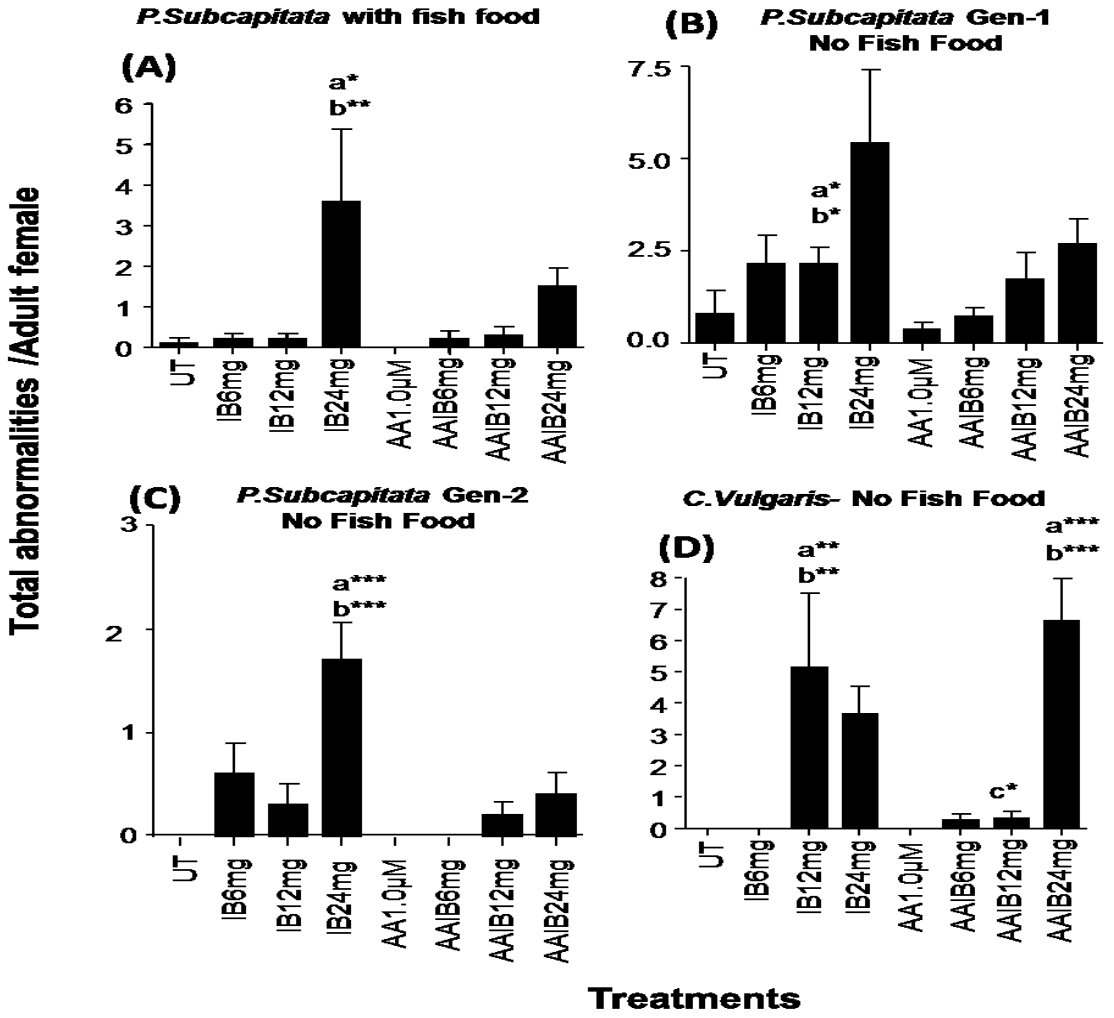


Figure. 4.3: Developmental toxicity of ibuprofen in *Daphnia* on arachidonic acid (AA) poor and rich diets: Reproductively mature *D. magna* were acclimatized to different diets that vary in AA; and fecundity determined during ibuprofen, AA, or combination treatments. Stock *P. subcapitata* and fish food (A), acclimatized to *P. subcapitata* for one (B) or two generations (C) and *Chlorella vulgaris* (Low in AA) (D). Total number of abnormal neonates produced in a 10 d period or from four broods was quantified. Superscript (a) indicates significant difference in neonates produced from untreated, a (b) indicates significant difference from AA treatment and a (c) indicates a significant difference from IB and corresponding IB+AA treatments as determined by one-way ANOVA followed by Tukey's multiple comparison test (* = $p < 0.05$), (** = $p < 0.01$) (*** = $p < 0.001$). Data are shown as mean ± SEM.

We also considered the likelihood of ibuprofen causing non-lethal developmental defects. Because of the differences in reproduction between the dietary groups, we examined the percent of neonates that showed non-lethal abnormalities. *D.magna* on a moderately AA rich diet produced less than 0.2% non-lethal abnormalities (defective secondary antenna or apical spine) (Figure.4.4A). *Daphnia* in first generation without fish food showed a significantly higher percent of non-lethal abnormalities with about 12% abnormal neonates at 24 mg L^{-1} ibuprofen (Figure. 4.4B). However, the non-lethal abnormalities completely disappeared in the second generation of *D. magna* fed *P. subcapitata* without fish food (Figure. 4. 4). Ibuprofen caused about 6% of the neonatal daphnids to form developmental defects, when fed *C. vulgaris* (Figure.4.4D). AA supplementation successfully recovered the non-lethal abnormalities observed at 12 mg L^{-1} ibuprofen exposure when fed low AA diet *C. vulgaris* (Figure. 4. 4D) AA also mitigated the developmental toxicity induced by 24 mg L^{-1} ibuprofen when fed *P. subcapitata* (Figure. 4.4B). Appendix.D.2 shows the number of individual neonates with non-lethal developmental defects following ibuprofen exposure or AA mitigation under the different diets.

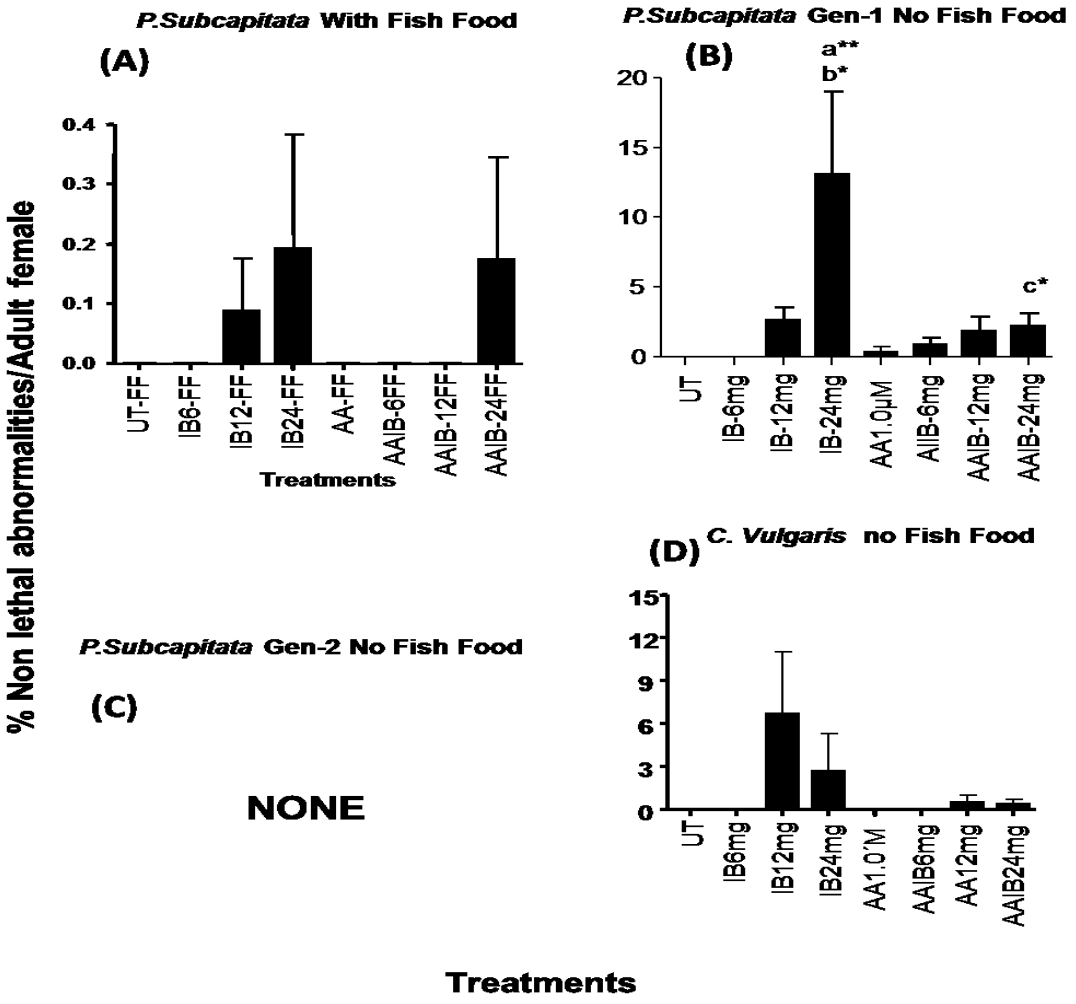


Figure. 4.4: Percent non-lethal abnormalities produced by ibuprofen exposure: Reproductively mature *D. magna* were acclimatized to different diets that vary in AA; and fecundity determined during ibuprofen, AA, or combination treatments. Stock *P. subcapitata* and fish food (A), acclimatized to *P. subcapitata* for one (B) or two generations (C) and *Chlorella vulgaris* (Low in AA) (D). Percent non-lethal abnormalities produced in a 10 d period or from four broods were quantified. Superscript (a) indicates significant difference in neonates produced from untreated, a (b) indicates significant difference from AA treatment and a (c) indicates a significant difference from IB and corresponding IB+AA treatments as determined by one-way ANOVA followed by Tukey's multiple comparison test (* = $p < 0.05$), (** = $p < 0.01$) (*** = $p < 0.001$). Data are shown as mean ± SEM.

4.5 Discussion

Ibuprofen at 6 to 24 mg L^{-1} caused no reproductive toxicity in a typical diet. However, ibuprofen caused a concentration dependent (6 mg L^{-1} to 24 mg L^{-1}) reduction in *D. magna* fecundity in daphnids fed *C. vulgaris* that contain significantly lower amounts of AA (Figure. 4. 1). In addition, *C.vulgaris* is also low in DHA and EPA (Guedes et al., 2011), which are the other PUFAs important for reproduction in *Daphnia* (Brett and Muller-Navarra, 1997; Ravet et al., 2012). Therefore, the lack of these key polyunsaturated fatty acids may also perturb daphnid reproduction when fed *C. vulgaris*; although it is interesting to note that AA supplementation did induce significant recovery from ibuprofen toxicity. Thus, the drop in fecundity induced by ibuprofen might be due to inhibition of AA metabolite synthesis as this is the mechanism of action of ibuprofen (Bancos et al., 2009). Overall, dietary AA or its metabolites appear to be crucial for *D. magna* reproduction and the adverse influence of toxicants becomes severe when the diet is deficient of AA.

Further corroboration of the beneficial effects of AA on *D.magna* reproduction is provided by the observation that AA supplementation induces recovery of fecundity lost during ibuprofen exposure in daphnids provided a diet low in AA (Figure. 4.1D). Further, daphnids recently switched from a normal diet to a diet without fish food, which probably contains AA and other sources of dietary fatty acids from the fish meal, also showed recovery in fecundity following AA supplementation (Figure. 4.1B). This finding indicates that AA, which normally is accumulated in the daphnid ovary as it nears maturity (Goulden and Place, 1993), is in part responsible for reproduction, and

ibuprofen as a cyclooxygenase inhibitor most likely works by perturbing metabolism or function of AA. Furthermore, the data indicate that as diet degrades in the environment, daphnids (and other species) will become more susceptible to the toxic effects of chemicals such as ibuprofen.

Ibuprofen induced significant lethal and non-lethal developmental defects. These defects included reduced digits; shorter length of the digits, shortened flagellum on secondary antenna, or curved apical spines (Figure. 4. 2). The lethal abnormalities include aborted eggs, and neonates that are born dead. Developmental defects in *D.magna* are concentration dependent similar to those found in *Danio rerio* (David and Pancharatna, 2009).

The non-lethal abnormalities include developmental defects in secondary antenna and apical spine deformities that are comparable to the tailless (tll gene knockout phenotypes) in *Drosophila* (Gui et al., 2011) or dll Knockout phenotypes in *Daphnia* (Kato et al., 2011). Once again, *D. magna* provided a low AA diet are most sensitive to the developmental defects of ibuprofen suggesting that the developmental perturbations of ibuprofen are elicited through its inhibition of AA metabolism. However, the beneficial effects of AA supplementation are limited to only the 12 mg L^{-1} ibuprofen group; and therefore the developmental results are not as strong as the results observed for fecundity. However, a trend was observed in which AA supplementation reduced developmental toxicity in each group, even if the data was not statistically significant in each group. Ibuprofen is a commonly used NSAID drug that blocks eicosanoid synthesis by inhibiting cyclooxygenase enzymes and reduces inflammation (Bancos et al., 2009).

Therefore, exposing *Daphnia* to ibuprofen is expected to cause eicosanoid synthesis inhibition from AA. Eicosanoids play important roles in arthropod reproduction. The *Daphnia* genome is found to possess at least one isoform of cyclooxygenase (COX) genes (NCBI accession number EFX85708.1) (Colbourne et al., 2011) to indicate that *Daphnia* have the machinery to synthesize eicosanoids and they use eicosanoids for physiological purposes possibly in reproduction. Interestingly, ibuprofen was unable to disrupt reproduction in *Daphnia* that are on the moderately AA rich diet (*P. subcapitata*) along with fish food supplementation (Figure.4. 1A). Supplementation of AA with 24 mg L^{-1} ibuprofen caused lower fecundity that might be due to additive toxicity. However, daphnids provided a low AA diet appeared not to have the AA available to outcompete cyclooxygenase inhibition provided by the ibuprofen, and therefore showed lower fecundity and a larger percentage of developmental defects.

Our work demonstrates how cyclooxygenase inhibitors could potentially reduce fecundity and induce adverse developmental abnormalities of the antenna in *D. magna.* Further, it highlights the ability of a proper diet and specifically AA, in protection from reproductive and developmental toxicity caused by the cycloogenase inhibitor ibuprofen. These results also confirm our earlier results that AA is a key polyunsaturated fatty acid (PUFA) for reproduction and suggest that AA is a key PUFA for development. However, the concentrations of ibuprofen used are not environmentally relevant (Kolpin et al., 2002). In summary, ibuprofen induces reproductive and developmental defects and these defects are in part repressed by AA supplementation, suggesting that ibuprofen toxicity is mediated through an AA metabolic pathway such as cyclooxygenase in *D. magna*.

4.6 References

Ahlgren, G., Lundstedt, L., Brett, M., Forsberg, C., 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. J Plankton Res 12 809-818

Baldwin, W.S., Bailey, R., Long, K.E., Klaine, S., 2001. Incomplete ecdysis is an indicator of ecdysteroid exposure in *Daphnia magna*. Environ Toxicol Chem 20, 1564-1569.

Bancos, S., Bernard, M.P., Topham, D.J., Phipps, R.P., 2009. Ibuprofen and other widely used non-steroidal anti-inflammatory drugs inhibit antibody production in human cells. Cellular Immunology 258, 18-28.

Bec, A., Desvilettes, C., Vera, A., Lemarchand, C., Fontvieille, D., Bourdier, G., 2003. Nutritional quality of a freshwater heterotrophic flagellate: Trophic upgrading of its microalgal diet for *Daphnia hyalina*. Aquat Microb Ecol 32, 203-207.

Brett, M.T., Muller-Navarra, D.C., 1997. The role of highly unsaturated fatty acids in aquatic food web processes. Freshwater Biol 38, 483-499.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151, 315-331.

Buser, H.-R., Poiger, T., Müller, M.D., 1999. Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and in wastewater. Environmental Science & Technology 33, 2529-2535.

Carpenter, S., Kitchell, J., Hodgson, J., Cochran, P., Elser, J., Elser, M., Lodge, D., Kretchmer, D., He, X., Von, E., 1987. Regulation of lake primary productivity by food web structure. Ecology 68 1863-1876.

Colbourne, J.K., Pfrender, M.E., Gilbert, D., Thomas, W.K., Tucker, A., Oakley, T.H., Tokishita, S., Aerts, A., Arnold, G.J., Basu, M.K., Bauer, D.J., Caceres, C.E., Carmel, L., Casola, C., Choi, J.H., Detter, J.C., Dong, Q.F., Dusheyko, S., Eads, B.D., Frohlich, T., Geiler-Samerotte, K.A., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E.V., Kultz, D., Laforsch, C., Lindquist, E., Lopez, J., Manak, J.R., Muller, J., Pangilinan, J., Patwardhan, R.P., Pitluck, S., Pritham, E.J., Rechtsteiner, A., Rho, M., Rogozin, I.B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, Z.J., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y.I., Yamagata, H., Yamada, T., Ye, Y.Z., Shaw, J.R., Andrews, J., Crease, T.J., Tang, H.X., Lucas, S.M., Robertson, H.M., Bork, P., Koonin, E.V., Zdobnov, E.M., Grigoriev, I.V.,

Lynch, M., Boore, J.L., 2011. The Ecoresponsive genome of *Daphnia pulex*. Science 331, 555-561.

David, A., Pancharatna, K., 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). Environ Toxicol Pharmacol 27, 390-395.

Doll, T.E., Frimmel, F.H., 2003. Fate of pharmaceuticals-photodegradation by simulated solar UV-light. Chemosphere 52, 1757-1769.

Gaedke, U., Straile, D., 1998. Daphnids: Keystone species for the pelagic food web structure and energy flow - A body size-related analysis linking seasonal changes at the population and ecosystem levels. Advance Limnol 53, 587-610.

Ginjupalli, G., Baldwin, W., 2013. The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on *Daphnia magna* reproduction. Chemosphere 92, 1260-1266.

Ginjupalli, G., Baldwin, W.S., submitted. Arachidonic acid enhances reproduction in *Daphnia* and mitigates changes in sex ratios induced by pyriproxyfen Ectoxicol Environ Safety. submitted.

Goulden, C.E., Place, A.R., 1993. Lipid accumulation and allocation in daphnid cladocera. Bull Marine Sci 53, 106-114.

Guedes, A.C., Amaro, H., Barbosa, C., Pereira, R., Malcata, F., 2011. Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses. Food Res Int 44, 2721-2729.

Gui, H., Li, M.L., Tsai, C., 2011. A Tale of tailless. Dev Neurosci 33, 1-13.

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog Lipid Res 45, 160-186.

Han, G.H., Hur, H.G., Kim, S.D., 2006. Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: Occurrence and toxicity to *Daphnia magna*. Environ Toxicol Chem 25, 265-271.

Han, S., Choi, K., Kim, J., Ji, K., Kim, S., Ahn, B., Yun, J., Khim, J.S., Zhang, X.W., Giesy, J.P., 2010. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquat Toxicol 98, 256-264.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Res 42, 3498-3518.

Kim, S., Cho, J., Kim, I., Vanderford, B., Snyder, S., 2007. Occurence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. Water Res 41, 1013 – 1021.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environ Sci Tech 36, 1202-1211.

Kosjek, T., Heath, E., Krbavčič, A., 2005. Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples. Environ Int 31, 679-685.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P., Tandler, A., 2001. The effect of dietary arachidonic acid (20:4ω−6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture 193, 107-122.

Koven, W.M., Tandler, A., Kissil, G.W., Sklan, D., 1992. The importance of ω-3 highly unsaturated fatty acids for growth in larval *Sparus aurata* and their effect on survival, lipid composition and size distribution. Aquaculture 104, 91-104.

Lee, C.S., 2003. Biotechnological advances in finfish hatchery production: A review. Aquaculture 227, 439-458.

Miller, W., Geeene, J., Shiroyama, T., 1978. The *Selenastrum capricornutum* printz algal assay bottle test.Experimental design, application, and data interpretation protocol. Environmental Research Laboratory, Corvalis,Oregon,97330, pp. 11-26.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proceedings of the National Academy of Sciences of the United States of America 90, 11693-11697.

Phelps, R., 2010. Recent advances in fish hatchery management. R. Bras. Zootec. 39, 95-101.

Pounds, N., Maclean, S., Webley, M., Pascoe, D., Hutchinson, T., 2008. Acute and chronic effects of ibuprofen in the mollusc *Planorbis carinatus* (Gastropoda: Planorbidae). Ecotoxicol Environ Safety 70, 47-52.

Ravet, J., Persson, J., Brett, M.T., 2012. Threshold dietary polyunsaturated fatty acid concentrations for *Daphnia pulex* growth and reproduction. Inland Waters 2, 199-209.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., Estevez, A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177, 191-199.

Støttrup, J., Jensen, J., 1990. Influence of algal diet on feeding and egg-production of the calanoid copepod *Acartia tonsa Dana*. J. Exp. Mar. Biol. Ecol 141, 87-105.

Taipale, S.J., Kainz, M.J., Brett, M.T., 2011. Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids in *Daphnia*. Oikos 120, 1674-1682.

Uslu, M.O., Jasim, S., Arvai, A., Bewtra, J., Biswas, N., 2013. A Survey of occurrence and risk assessment of pharmaceutical substances in the great lakes basin. Ozone-Science & Engineering 35, 249-262.

Wacker, A., Martin-Creuzburg, D., 2007. Allocation of essential lipids in *Daphnia magna* during exposure to poor food quality. Funct Ecol 21, 738-747.

Wang, H.Y., Olmstead, A.W., Li, H., LeBlanc, G.A., 2005. The screening of chemicals for juvenoid-related endocrine activity using the water flea *Daphnia magna*. Aquat Toxicol 74, 193-204.

Yanes-Roca, C., Rhody, N., Nystrom, M., Main, K.L., 2009. Effects of fatty acid composition and spawning season patterns on egg quality and larval survival in common snook (*Centropomus undecimalis*). Aquaculture 287, 335-340.

Zuo, R.T., Ai, Q.H., Mai, K.S., Xu, W., Wang, J., Xu, H.G., Liufu, Z.G., Zhang, Y.J., 2012. Effects of dietary docosahexaenoic to eicosapentaenoic acid ratio (DHA/EPA) on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). Aquaculture 334, 101-109.

CHAPTER FIVE

DISCUSSION

Daphnia play crucial roles in aquatic ecosystems as mid-level consumers that feed on algae, bacteria, protozoans and detritus and in turn are a source of food for large invertebrates and fish (Gaedke and Straile, 1998; Tessier and Woodruff, 2002). In turn they are essential in the trophic transfer of essential nutrients and a key indicator species (Gaedke and Straile, 1998; Tessier and Woodruff, 2002). *Daphnia* are also widely used in toxicity testing due to their high sensitivity to a broad range of chemicals, small size, short life-cycles and ease of culturing in the laboratory (Vandenbrouck et al., 2010). Under favorable environmental and dietary conditions, an all-female population of *Daphnia* can build huge populations in a short span through clonal expansion (Anderson and Jenkins, 1942).

As primary consumers of algae *Daphnia* build rich populations in short time that can keep algal blooms under check (Wilson and Chislock, 2013). However, under stressful environmental conditions such as overcrowding (Smith et al., 2009), reduced photoperiods (Deng and Lynch, 1996) or scarcity of food, the population of *Daphnia* decreases as they resort to sexual mode of reproduction involving males that do not reproduce (Olmstead and LeBlanc, 2002; Tatarazako et al., 2003). Though sexual reproduction affects population growth, it is thought to impart evolutionary fitness to *Daphnia* through genetic recombination and production of resting eggs to prevent population extinction (Ebert, 2005).

In addition to the environmental stressors, certain anthropogenic chemicals such as pyriproxyfen released into the aquatic environment induce male production and reduce *Daphnia* fecundity that might have negative impacts on the ecosystem as a whole. Previous research indicated that pyriproxyfen, a commonly used juvenile hormone analog insecticide, found at 300pM (Olmstead and LeBlanc, 2003) concentration in the environment is non-toxic to human beings (Sullivan and Goh, 2008), but causes reproductive toxicity in *Daphnia* by inducing male production without affecting survival. My research is important in characterizing the reproductive toxicity to *Daphnia* in terms of concentration, age and time dependent effects of pyriproxyfen hitherto unknown.

Objective-1: Determine the reproductive, male production and temporal effects of pyriproxyfen on *Daphnia magna*.

The objective -1 is aimed at characterizing the off target effects of pyriproxyfen in *D.magna* in an age, time and concentration-dependent fashion. We used a series of modified chronic toxicity assays using *D. magna* and quantified the male production effects of pyriproxyfen in acute and continuous and periodic exposures at in juvenile and adolescent/adult age groups.

5.1 New findings (objective-1)

- Pyriproxyfen increases male production in a concentration-dependent fashion with an EC50 of 156 pM (50.24 ng L^{-1}) and a LOEC of 78 pM (25ngL^{-1}) within

the range of environmentally relevant concentrations 170 - 310 pM (55 - 100ng/L) (Olmstead and LeBlanc, 2003; Matsumoto et al., 2008).

- Pyriproxyfen decreased overall fecundity in 7, 14, 21-d old female parthenogenic daphnids.
- Longer pyriproxyfen exposures (8–12 d) extend male production and decrease reproduction.
- Daphnids exposed for shorter durations such as 2–4 d recover from pyriproxyfen toxicity quickly and produce a relatively normal abundance of neonates, especially adults.
- However, juvenile daphnids (3-d) are very sensitive to pyriproxyfen. The primary effect on juvenile daphnids is reduced reproduction and protracted development; not male production.

The findings of this study supported our hypothesis. However, the adverse effects on 3-d old *Daphnia* in terms of delayed maturity and irreversible loss of fecundity due to prolonged exposures are unexpected and the mechanism for delayed maturity is not known. Based on these new findings from this study, we recommend that multiple sprayings of pyriproxyfen spanning less than 7 day intervals around water bodies needs due caution in terms of its potential adverse effects with significant developmental delays in younger daphnids and male production effects in mature daphnids compounded by repeated exposures.

Objective-2: Test whether the HR97g inhibitor, arachidonic acid, blocks male production.

The findings from the study in objective-1 with regard to temporal and age dependent recovery in *Daphnia* from the reproductive toxicity of pyriproxyfen, lead us to objective-2. Therefore, we decided to explore the potential factors that contributed for the recovery. We hypothesized that the ability of *Daphnia* in recovering from the toxicity of pyriproxyfen in part depends on the quality and quantity of diet. We specifically hypothesized that AA is preferentially accumulated in the daphnid ovary as they become sexually mature (Ahlgren et al., 1990; Goulden and Place, 1993; Bec et al., 2003; Wacker and Martin-Creuzburg, 2007; Taipale et al., 2011), because it influences daphnia fecundity and probably male production based on our results indicating that the receptor HR97 found in ovaries is inhibited by AA. It is interesting to note that *Daphnia* concentrate EPA and AA from phytoplankton diet and preferentially accumulate these fatty acids in the ovaries (Ahlgren et al., 1990; Goulden and Place, 1993; Bec et al., 2003; Wacker and Martin-Creuzburg, 2007; Taipale et al., 2011). *P. subcapitata,* our typical algal diet contains moderately rich levels of AA (Brown et al., 1997; Guedes et al., 2011), N. oculata contains high levels of AA, and *C. vulgaris* is a diet with low levels of AA (ref). Therefore, we tested how *D.magna* treated with 155pM or 310pM pyriproxyfen were affected while being concomitantly exposed to AA and neonatal sex ratios were quantified.

In addition, we decided to test how AA, one of the two fatty acids accumulated in the ovary affects overall *D. magna* reproduction. We hypothesized that providing an algal diet that contains low levels of AA will decrease reproduction through the use of these diets and supplementing diets with AA.

5.2 New findings (objective-2)

Out of the fatty acids tested, only AA affected male production in presence of pyriproxyfen although AA only altered male production when daphnids were fed half the regular diet.

- Low AA (*C. vulgaris*) diets resulted in lower fecundity.
- AA supplementation of *P. subcapitata* 1.0μM or 4.0 μM (Regular diet 6million cells/day) in presence of 155pM or 310PM pyriproxyfen partially recovered the neonatal sex ratios and fecundity.
- Arachidonic acid supplementation of low AA diet (*C. vulgaris*) enhanced reproduction.
- Low AA diets result in higher male production and AA supplementation of these diets show partial recovery in neonatal sex ratios and fecundity.
- *D. magna* exposed during a diet switch that were previously on a high AA diet show a phenotype typical of a underfed daphnid fed *P. subcapitata* when investigating sex ratios (higher female production) and not a *C. vulgaris* (higher male production) diet This might be due to greater AA accumulation from the diet during earlier life stages.

Overall, the findings of this study support our hypothesis that AA is accumulated in the ovary for reproductive purposes. However, AA supplementation of a diet that is moderately rich to support normal reproduction doesn't provide any additional benefit. We hypothesize that there is a threshold AA concentration required to support normal reproduction and it is met through a moderate AA diet, and therefore supplementation had no effect. However, the beneficial effects of dietary AA or AA supplementation are clearly evident under half-fed and low AA diets with or without pyriproxyfen. In summary, AA is a key fatty acid in reproduction, provides protection from pyriproxyfen-mediated male production, and AA enrichment may be beneficial in some circumstances. This last point may prove beneficial when farming economically important decapod crustaceans.

Objective-3: Determine the adverse developmental effects of the eicosanoid synthesis inhibitor Ibuprofen in *Daphnia.*

We further decided to determine whether perturbations in AA metabolism reduce reproduction as our previous work indicated the beneficial effects of AA on reproduction. Therefore, we exposed the *Daphnia* to ibuprofen, a commonly used anti-inflammatory pharmaceutical compound that inhibits the formation of eicosanoids by inhibiting cyclooxygenase (Bancos et al., 2009). Considering the physiological roles played by AA and its metabolites in survival and reproduction in several different species, we hypothesized ibuprofen toxicity would in part be mitigated by AA due to its ability to

out-compete cyclooxygenase inhibition. Interestingly, AA metabolism pathways are enhanced in *D. pulex* relative to most other species (Colbourne et al., 2011).

The purpose of this study is to determine the specific reproductive and developmental effects of ibuprofen on adult *D. magna,* and the potential role of AA in mitigating the toxicity of ibuprofen. Overall, this study is useful in providing a mechanistic basis for the enhanced reproduction observed in AA supplementation of the low AA diets.

5.3 New findings (objective-3)

- Ibuprofen caused a concentration dependent (6mg/L to 24mg/L) reduction in *D. magna* fecundity in daphnids fed *C.vulgaris* that has significantly lower amounts of AA.
- This is probably due to ibuprofen as a cyclooxygenase inhibitor.
- AA supplementation to *C. vulgaris* diet was able to overcome the inhibitory action of ibuprofen.
- Dietary AA or its metabolites are crucial for *D. magna* reproduction and the adverse effects of ibuprofen are much more severe when the diet is deficient of AA.
- Additional to the adverse effects on fecundity, ibuprofen induced concentration dependent lethal and non-lethal developmental abnormalities in *Daphnia*.
- The lethal abnormalities include aborted eggs and neonates that are born dead.

- The non-lethal developmental defects include shortened swim hair and digits on the secondary antenna and apical spine deformities that are comparable to the tailless in *Drosophila* (Gui et al., 2011).
- Similar to effects on fecundity, the daphnids provided a low AA diet are most sensitive to the developmental defects of ibuprofen suggesting that the developmental perturbations of ibuprofen are elicited through its inhibition of AA metabolism.

Interestingly, ibuprofen did not disrupt reproduction in *Daphnia* that are fed a moderately AA rich diet (*P. subcapitata*) along with fish food supplementation (Figure.1A). Overall, the findings of our study supported our hypothesis. Ibuprofen induced concentration dependent drop in fecundity coupled with lethal and non-lethal developmental deformities in daphnids fed on low AA diets. The adverse effects of ibuprofen were overcome by AA supplementation that might have revered the cyclooxygenase dependent inhibition of AA and its metabolite synthesis by ibuprofen. However, AA supplementation has limited beneficial effect in reversing the adverse effects at 24mg/L ibuprofen. In addition, AA supplementation to moderately rich diet did not provide any additional benefit similar to the finding in objective -2.

5.4 Purpose of this research

This bigger picture of my research include providing additional details regarding age, concentration and time dependent off target adverse effects of pyriproxyfen, a commonly used juvenile hormone analog insecticide that is considered safe for human beings. We came up with recommendation regarding safer use of the pesticide near aquatic bodies where daphnids play key roles in the food web sustenance. We found that arachidonic acid, an ω-6 PUFA accumulated in daphnid ovary has roles in reproduction and recommended that AA supplementation of the diet has the potential to support reproduction and provided protection from toxic compounds such as pyriproxyfen. Finally we provided the possible mechanistic basis for reproductive and developmental toxicity induced by ibuprofen, a commonly found anti-inflammatory compound in aquatic bodies.

5.5 References

Ahlgren, G., Lundstedt, L., Brett, M., Forsberg, C., 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. J Plankton Res 12 809-818

Anderson, B., Jenkins, J., 1942. A Time Study of Events in the Life span of *Daphnia magna*. Biol Bull 83, 260-272.

Bancos, S., Bernard, M.P., Topham, D.J., Phipps, R.P., 2009. Ibuprofen and other widely used non-steroidal anti-inflammatory drugs inhibit antibody production in human cells. Cellular Immunology 258, 18-28.

Bec, A., Desvilettes, C., Vera, A., Lemarchand, C., Fontvieille, D., Bourdier, G., 2003. Nutritional quality of a freshwater heterotrophic flagellate: Trophic upgrading of its microalgal diet for *Daphnia hyalina*. Aquat Microb Ecol 32, 203-207.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151, 315-331.

Colbourne, J.K., Pfrender, M.E., Gilbert, D., Thomas, W.K., Tucker, A., Oakley, T.H., Tokishita, S., Aerts, A., Arnold, G.J., Basu, M.K., Bauer, D.J., Caceres, C.E., Carmel, L., Casola, C., Choi, J.H., Detter, J.C., Dong, Q.F., Dusheyko, S., Eads, B.D., Frohlich, T., Geiler-Samerotte, K.A., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E.V., Kultz, D., Laforsch, C., Lindquist, E., Lopez, J., Manak, J.R., Muller, J., Pangilinan, J., Patwardhan, R.P., Pitluck, S., Pritham, E.J., Rechtsteiner, A., Rho, M., Rogozin, I.B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, Z.J., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y.I., Yamagata, H., Yamada, T., Ye, Y.Z., Shaw, J.R., Andrews, J., Crease, T.J., Tang, H.X., Lucas, S.M., Robertson, H.M., Bork, P., Koonin, E.V., Zdobnov, E.M., Grigoriev, I.V., Lynch, M., Boore, J.L., 2011. The Ecoresponsive genome of *Daphnia pulex*. Science 331, 555-561.

Deng, H., Lynch, M., 1996. Change of genetic architecture in response to sex. Genetics 143 203-212.

Ebert, D., 2005. Ecology, epidemiology, and evolution of parasitism in *Daphnia*. National Center for Biotechnology Information, Bethesda, MD.

Gaedke, U., Straile, D., 1998. Daphnids: Keystone species for the pelagic food web structure and energy flow - A body size-related analysis linking seasonal changes at the population and ecosystem levels. Advance Limnol 53, 587-610.

Goulden, C.E., Place, A.R., 1993. Lipid accumulation and allocation in daphnid cladocera. Bull Marine Sci 53, 106-114.

Guedes, A.C., Amaro, H., Barbosa, C., Pereira, R., Malcata, F., 2011. Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses. Food Res Int 44, 2721-2729.

Gui, H., Li, M.L., Tsai, C., 2011. A Tale of tailless. Dev Neurosci 33, 1-13.

Matsumoto, T., Ikuno, E., Itoi, S., Sugita, H., 2008. Chemical sensitivity of the male daphnid, *Daphnia magna*, induced by exposure to juvenile hormone and its analogs. Chemosphere 72, 451-456.

Olmstead, A.W., LeBlanc, G.A., 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. J Exp Zool 293, 736-739.

Olmstead, A.W., LeBlanc, G.A., 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environ Health Perspect 111, 919-924.

Smith, A., Acharya, K., Jack, J., 2009. Overcrowding, food and phosphorus limitation effects on ephippia production and population dynamics in the invasive species *Daphnia lumholtzi*. Hydrobiologia 618, 47-56.

Sullivan, J., Goh, K., 2008. Environmental fate and properties of pyriproxyfen. J Pest Sci 33, 339-350.

Taipale, S.J., Kainz, M.J., Brett, M.T., 2011. Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids in *Daphnia*. Oikos 120, 1674-1682.

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., Iguchi, T., 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53, 827–833.

Tessier, A., Woodruff, P., 2002. Trading off the ability to exploit rich versus poor food quality. Ecol Let 5, 685-692.

Vandenbrouck, T., Jones, O., Dom, N., Griffin, J., De Coen, W., 2010. Mixtures of similarly acting compounds in *Daphnia magna*: From gene to metabolite and beyond. Environ Int 36, 254-268.

Wacker, A., Martin-Creuzburg, D., 2007. Allocation of essential lipids in *Daphnia magna* during exposure to poor food quality. Funct Ecol 21, 738-747.

Wilson, A., Chislock, M., 2013. Ecological control of cyanobacterial blooms in freshwater ecosystems. In : Ferrao Filho,A. (Ed.). Cyanobacteria: Ecol Toxicol Management. Nova Science Publishers, UK.

Appendix. A

Supplementary material to Chapter-2

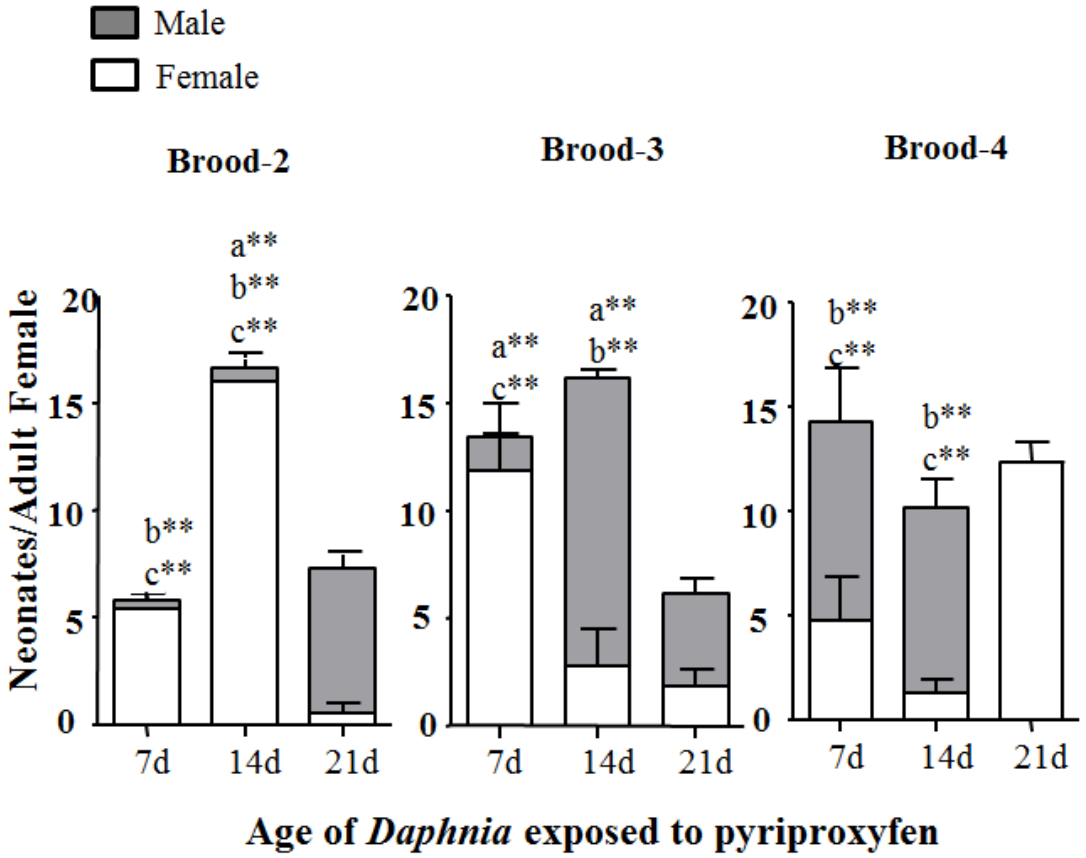


Figure: Appendix-A.1: Brood-dependent sensitivity to pyriproxyfen in *Daphnia magna*. Female *Daphnia magna* at 7, 14, and 21-days old were treated with 155 pM pyriproxyfen for 12 days or until four broods were produced. None of the untreated daphnids from any of the assays produced males (data not shown). Data are shown as mean $\pm$ SEM. (a) Indicates a significant difference in the number of neonates produced, (b) indicates a significant difference in the number of male neonates produced, and (c) indicates a significant difference in the number of female neonates produced compared to the 21-day old *Daphnia*. Statistical differences were analyzed by ANOVA followed by Dunnett's multiple comparison test. An (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$.

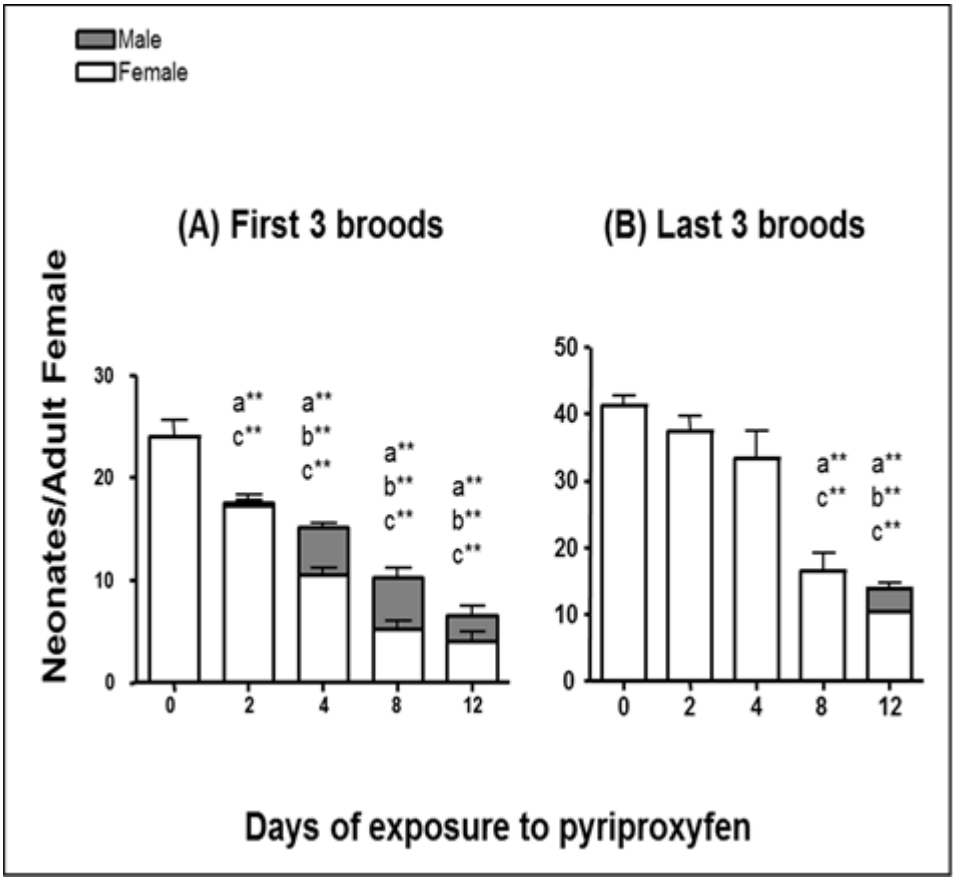


Figure. Appendix.A.2: Brood wise fecundity and sensitivity in male production in 3-day old *Daphnia magna*. Female *Daphnia magna* that were 3 days old were exposed to 155 pM (50 ng /L) pyriproxyfen for 0-12 days, and production of female and male neonates from the first three (A) and last three broods (B) per adult female were quantified. Data are shown as mean $\pm$ SEM. (a) Indicates a significant difference in the total number of neonates produced, (b) indicates a significant difference in the number of male neonates produced, and (c) indicates a significant difference in the total number of female neonates produced compared to the control. Statistical differences were analyzed by ANOVA followed by Dunnett's multiple comparison test and an (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$.

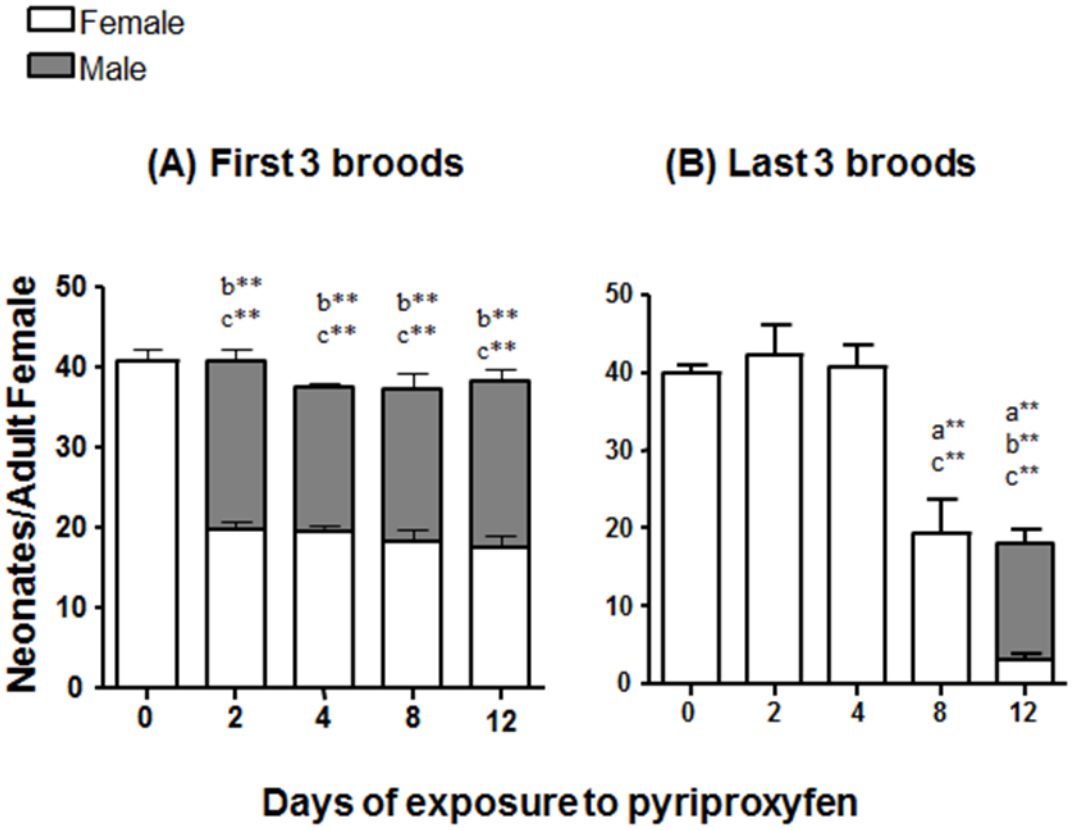


Figure. Appendix.A.3: Brood wise fecundity and sensitivity in male production in 10-day old *Daphnia magna*. Female *Daphnia magna* that were 10 days old were exposed to 155 pM (50 ng /L) pyriproxyfen for 0-12 days, and production of female and male neonates from the first three and last three broods per adult female were quantified. Data are shown as mean $\pm$ SEM. (a) Indicates a significant difference in the total number of neonates produced, (b) indicates a significant difference in the number of male neonates produced, and (c) indicates a significant difference in the total number of female neonates produced compared to the control. Statistical differences were analyzed by ANOVA followed by Dunnett's multiple comparison test and an (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$.

Appendix-B

Preamble to Chapter-3: Connection between HR97g and Arachidonic Acid.

A novel HR97 group of receptors recently identified in *Daphnia* contain three members namely HR97a, HR97b and HR97g (Colbourne et al., 2011). The age dependent expression pattern determined by qPCR demonstrates that in the early life stages of life (days 2-4), *Daphnia magna* express low levels of each HR97 receptor. However, as daphnids age the expression of HR97a and HR97b receptors increase with a peak expression at day 7 (Figure.4 A&B). HR97a and HR97b show nearly identical expression patterns (Figure.4 A&B). Interestingly, these receptors are found in tandem repeat (Thomson et al., 2009). However, differing from HR97a and HR97b, the expression of HR97g receptor continues to increase into adult hood and reaches a peak in mature *Daphnia magna* between days 7 to 14 (Figure.4C). Thus HR97g appears to have a different role than HR97a and HR97b in adult *Daphnia magna*.

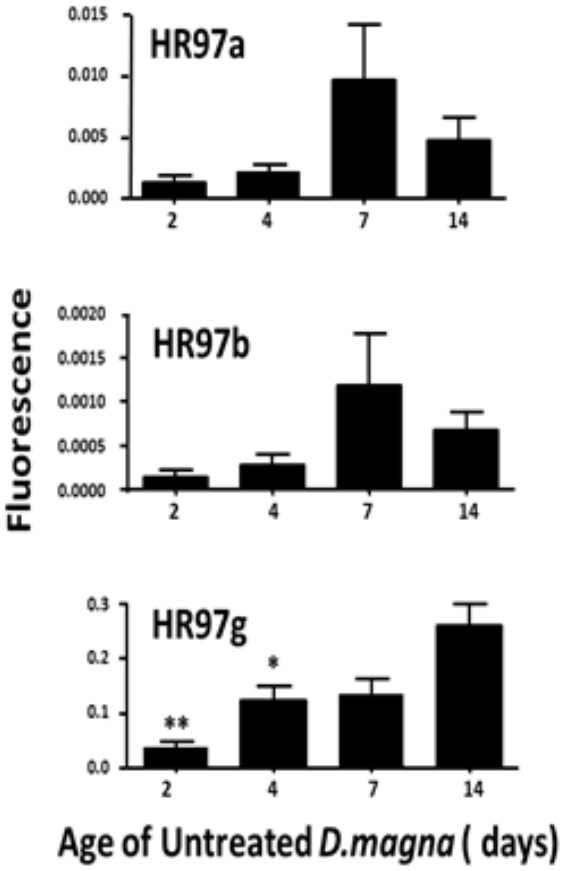


Figure. Appendix.B.1 Age dependent expression of Hr97a, HR97b and HR97g *Daphnia* nuclear receptors was determined by qPCR performed using cDNA made by MMLV reverse transcription method from RNA belonging to 2, 4, 7 and 14 days old *Daphnia magna*. Data are normalized to β-actin, a house keeping gene. Statistical differences in relative expression across ages were determined by Dunnett's post hoc test using Graph Pad prism 4.0

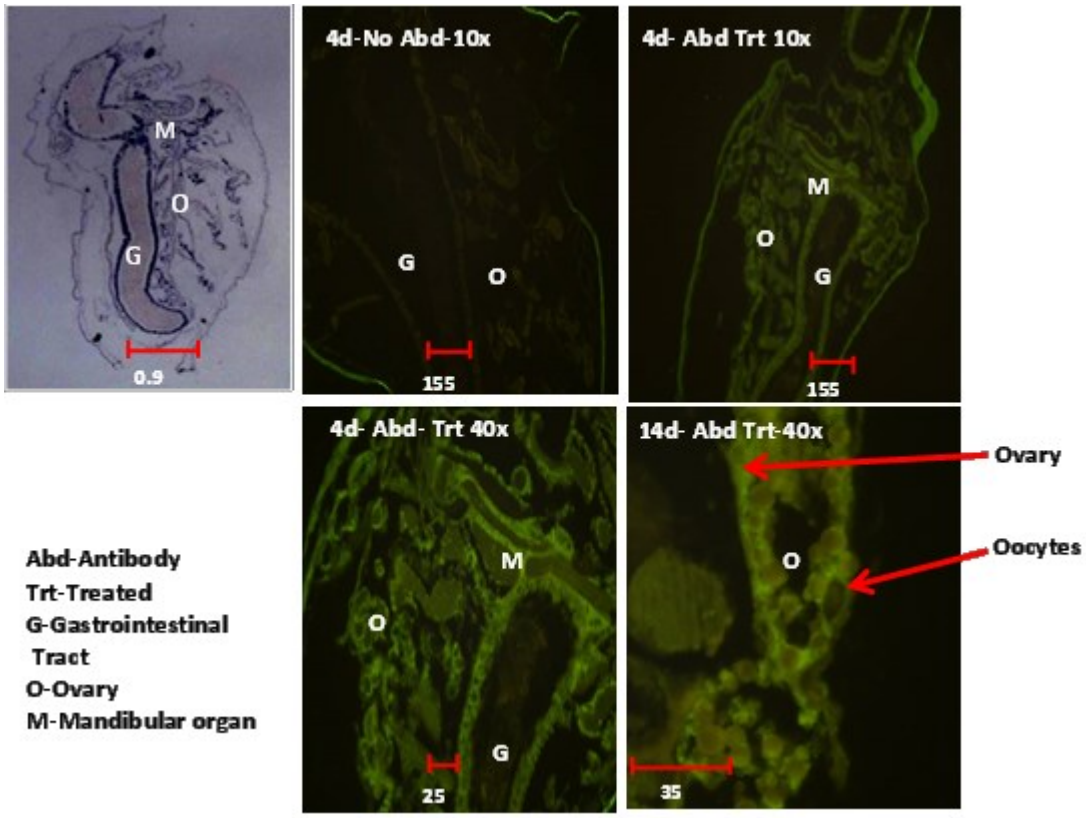


Figure.3.2.2 Immunohistochemical analysis was performed to determine tissue and organ specific expression of HR97g receptor. Antigen retrieval was performed on paraffin embedded micro sections of formaldehyde fixed *Daphnia magna*. Sections are treated with a primary antibody prepared against a KLH-conjugated N-terminal (A/B domain) sequence (GSSNEENAVPENKSC) of HR97g (Genscript) in rabbit. Secondary anti-rabbit antibody made in goat tagged to Alexafluor488 dye was used for antigen retrieval. Fluorescence emitted by the tissues imaged by Nikon fluorescent microscope. Fluorescence intensity is considered as proportional to the tissue specific expression of HR97g receptor. Micro sections that were not treated by primary antibody are used as negative controls for comparison. Sections from immature *D.magna* at day 4 at 10x magnification and 40 x magnifications were compared with mature *D.magna* at Day 14.

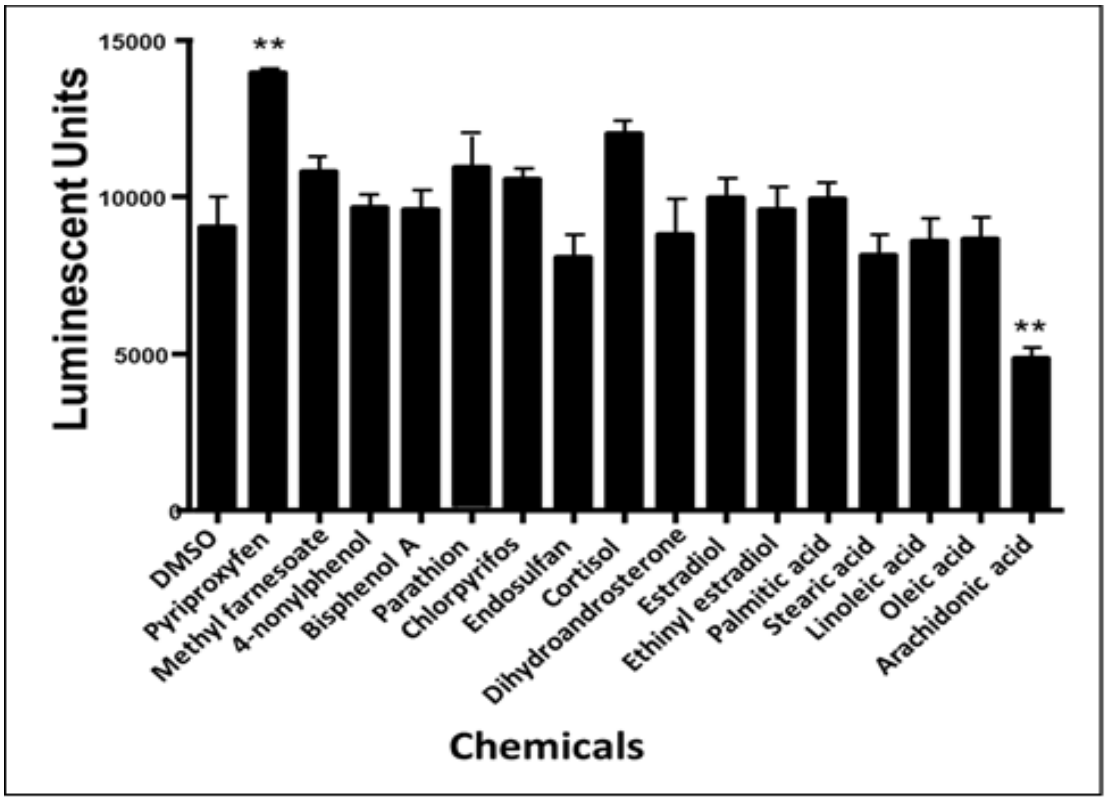


Figure.3.2.3 Chimeric genes containing *D.magna* HR97a (GAL4-97aDEF), HR97b (GAL4-97bDEF), or magna HR97g (GAL4-97gDEF) were attached to the DNA binding domain (DBD) of Gal4. Transactivation assays were performed in HepG2 cells to determine the potential ligands that could activate HR97g receptor in vitro. Ability of chemicals at 10μM belonging to different classes (Juvenile hormone analogs, industrial chemicals, commonly used pesticides, steroids hormones and dietary fatty acids) was determined. Activation of the receptor is quantified based on the luminescence determined by luminometer against the blank DMSO. Statistical significance was determined by ANOVA followed by Dunnett's or Tukey's multiple comparison post hoc tests using GraphPad Prizm 4.0 statistical package.

Appendix-C

Supplementary material to Chapter-3

Appendix C.Table.1: Effects of AA on survival in a standard 48 h acute toxicity test performed with neonate daphnids less than 24 h old.

AA concentration (μM)	Percent Survival
0	100
0.01	100
0.1	100
1.0	100
10	30*
100	0*

Statistical differences (shown with an asterisk) were determined by Fisher's exact test (http://www.vassarstats.net/).

Appendix-C Table.2: Effects of AA on reproduction of *D. magna* in a standard 21-day chronic toxicity test in first generation and 2nd generation daphnids.

	1st Generation	2nd Generation
AA concentration (μM)	Offspring/adult female[a]	Offspring/adult female[a]
0	83.2 ± 7.61	64.82 ± 7.20
1	80.5 ± 12.58	63.75 ± 9.90
2	79.36 ± 9.54	59.64 ± 8.99
4	80.18 ± 9.98	68.50 ± 8.95

[a] Data are presented as mean ± standard error. Statistical differences (shown with an asterisk) were determined by one-way ANOVA followed by Tukey's multiple comparison test.

Appendix-D

Supplementary material to Chapter-4

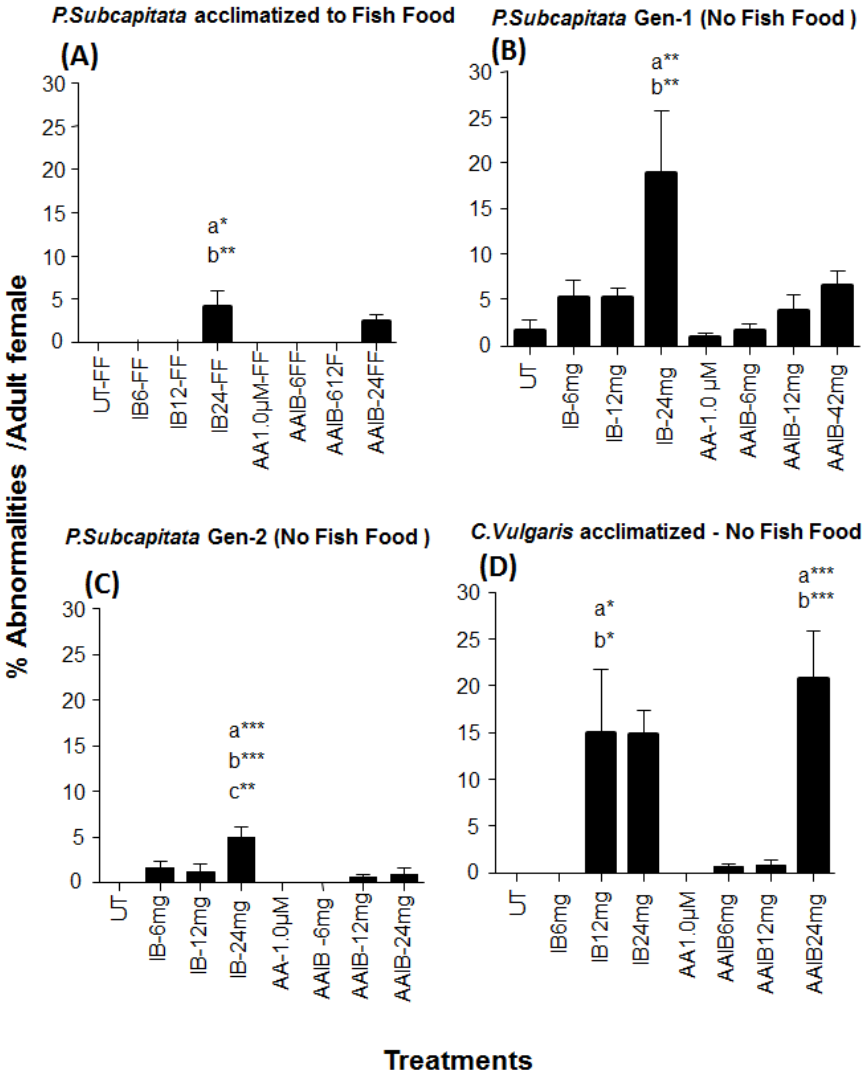


Figure.Appendix.D.1: % abnormal neonates in arachidonic acid (AA) poor and rich diets: Reproductively mature *D. magna* were acclimatized to different diets that vary in AA; and fecundity determined during ibuprofen, AA, or combination treatments. Stock *P. subcapitata* and fish food (A), acclimatized to *P. subcapitata* for one (B) or two generations (C) and *Chlorella vulgaris* (Low in AA) (D). Numbers of non-lethal abnormal neonates produced in a 10 d period or from four broods were quantified. Superscript (a) indicates significant difference in neonates produced from untreated, a (b) indicates significant difference from AA treatment and a (c) indicates a significant difference from IB and corresponding IB+AA treatments as determined by one-way ANOVA followed by Tukey's multiple comparison test (* = $p < 0.05$), (** = $p < 0.01$) (*** = $p < 0.001$). Data are shown as mean ± SEM

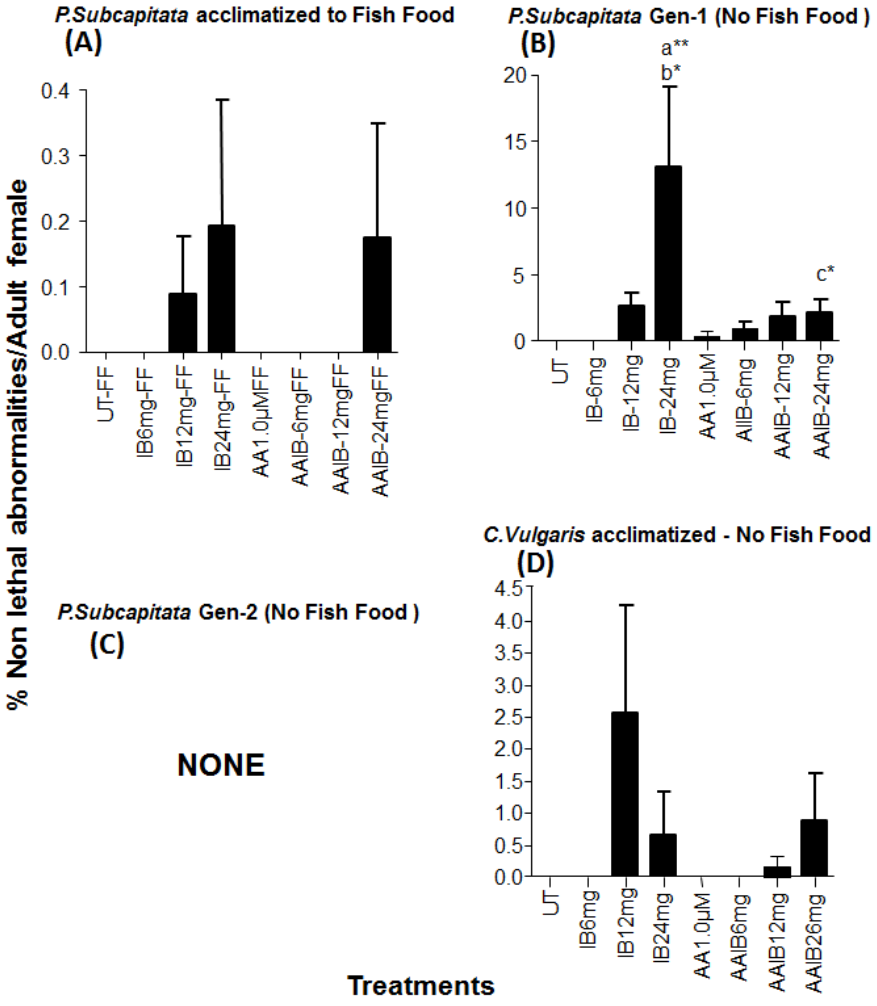


Figure. Appendix.D.2: Number of non-lethal abnormal neonates produced by ibuprofen exposure: Reproductively mature *D. magna* were acclimatized to different diets that vary in AA; and fecundity determined during ibuprofen, AA, or combination treatments. Stock *P. subcapitata* and fish food (A), acclimatized to *P. subcapitata* for one (B) or two generations (C) and *Chlorella vulgaris* (Low in AA) (D). Numbers of non-lethal abnormal neonates produced in a 10 d period or from four broods were quantified. Superscript (a) indicates significant difference in neonates produced from untreated, a (b) indicates significant difference from AA treatment and a (c) indicates a significant difference from IB and corresponding IB+AA treatments as determined by one-way ANOVA followed by Tukey's multiple comparison test (* = $p < 0.05$), (** = $p < 0.01$) (*** = $p < 0.001$). Data are shown as mean ± SEM